STUDIES ON ANTIBODIES AGAINST NUCLEIC ACID-FUROCOUMARIN PHOTOADDUCTS

ABSTRACT

THESIS SUBMITTED FOR THE DEGREE OF

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IN

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BY

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ABSTRACT

Psoralens are naturally occurring furocoumarins used in the treatment of skin disorders. They act by intercalating into DNA helix and form mono-and diadducts (crosslinks) with pyrimidines, especially thymidine, when irradiated with UV-A light (320-400 nm). The covalent interstrand crosslinks result in the blockage of replication and transcription activities. It is this ability to form interstrand crosslinks efficiently that established the basis for the use of psoralens as molecular probes for studying nucleic acid structure and function and DNA repair mechanism. The effect of resulting adduct (mono- or diadduct) on local structure of DNA depends on psoralen derivative. Unwinding of double helix, bent (kink) at the site of crosslinking and lengthening of DNA by an equivalent of about one base pair per bound psoralen molecule has been reported.

The present thesis describes the photomodification of calf thymus DNA and poly(dA-dT).poly(dA-dT) with 8-methoxypsoralen (8-MOP). Calf thymus DNA obtained commercially was purified free of protein and single stranded regions. The modification was studied by various physico-chemical techniques which include ultraviolet and fluorescence spectroscopy, thermal denaturation, nuclease S1 and Bal 31 sensitivity assays, hydroxyapatite chromatography and separation of bases on DEAE Sephadex A 50 matrix.

The photoadduct formation was indicated by changes in ultraviolet characteristics of modified nucleic acid polymers. The marked decrease in absorbance at 260 nm and significant hyperchromicity around 310 nm substantiates the formation of photoadduct. The substantial

fluorescence loss of 8-MOP upon UV-A irradiation in presence of DNA and poly(dA-dT).poly(dA-dT) coupled with a blue shift of 92 nm (i.e. from 507 nm to 415 nm) is a clear indication of the formation of photoadducts.

The photoaddition of 8-MOP to DNA and poly(dA-dT).poly(dA-dT) was analyzed by controlled melting of native and modified duplexes. The mid-point melting temperature (Tm) values of unmodified conformers were 78.5^{o}C (DNA) and 49.0^{o}C (poly(dA-dT).poly(dA-dT)). The Tm values of DNA-8MOP and poly(dA-dT)-8MOP photoconjugates were found to increase to the extent of 5.0^{o}C and 29.5^{o}C as compared to native conformers. Also, the percent hyperchromicity of either of the photoadducts (at 95^{o}C) was found to decrease. The increased melting temperature of photoadducts coupled with decreased percent hyperchromicity is characteristic of nucleic acid polymers containing covalent interstrand crosslinks. The data conclusively demonstrates that as a result of photoreaction between double stranded nucleic acids and 8-MOP, crosslinked species have been formed and it is this property which has conferred thermal stability to modified nucleic acids. Nuclease S1 resistance of denatured photoadducts (DNA-8MOP and poly(dA-dT)-8MOP) further substantiates the diadduct formation. Denatured DNA and poly(dA-dT).poly(dA-dT) were completely digested by nuclease S1.

On hydroxyapatite column, denatured DNA-8MOP and poly(dA-dT)-8MOP photoadducts showed major elution in the molarity region of double stranded DNA. This property of DNA-8MOP and poly(dA-dT)-8MOP could be attributed to the presence of covalent interstrand crosslinks. Furthermore, Bal 31 nuclease was found to be ineffective on DNA-8MOP crosslink (diadduct). DNA without modification was completely digested by the nuclease.

Acid hydrolysate of DNA-8MOP passed through DEAE Sephadex A 50 column revealed the modification of thymine to the extent of 69 percent.

The modification of nucleic acids by photoinduced addition of 8-MOP results in structural alteration ranging from subtle to profound. As a consequence these photoadducts became highly immunogenic in experimental animals in contrast to nDNA which is a poor immunogen. The DNA-8MOP and poly(dA-dT)-8MOP photoadducts induced precipitating antibodies in rabbit and goat, with a titer of >1:6400 and >1:51200 as revealed by direct binding ELISA. High titer of anti-poly(dA-dT)-8MOP antibodies suggests that poly(dA-dT)-8MOP photoadduct is a better immunogen as compared to DNA-8MOP, presumably due to the presence of excessive crosslinking sites.

Modified conformers along with unmodified polymers were used as competitive inhibitors to determine the specificity and selectivity of induced antibodies. The Sephadex G 200 purified anti-DNA-8MOP IgG showed a maximum of 86 percent inhibition in antibody-immunogen interaction. Fifty percent inhibition was seen at 0.16 ug/ml of immunogen. The maximum percent inhibition in antibody-immunogen interaction was increased further when antibodies affinity purified on a column of DNA-8MOP linked to polylysyl-Sepharose 4B matrix were used. Moreover, the competitor concentration required to achieve fifty percent inhibition was dropped significantly. The purified anti-DNA-8MOP antibodies did not show considerable cross reactivity with native DNA, and total buffalo thymus RNA, while some cross reactivity was observed with ssDNA. The affinity constant of anti-DNA-8MOP antibodies (1.13×10^{-9} M) together with above findings revealed the induction of immunogen specific antibodies.

Similarly induced antibodies against poly(dA-dT)-8MOP photoadduct exhibited high specificity for the immunogen as revealed by competitive binding and gel retardation assay. The altered electrophoretic mobility of anti-poly(dA-dT)-8MOP IgG- immunogen complex reiterates antibody binding to photoadduct. Quantitative precipitin data of anti-poly(dA-dT)-8MOP antibodies revealed an affinity constant of 6.80×10^{-10} M indicating high specificity of induced antibodies. DNA polymers of A-/ or B- conformation were found to be ineffective inhibitors of antibody- immunogen interaction. The binding specificity of anti-poly(dA-dT)-8MOP IgG with DNA-8MOP and DNA-psoralen photoadduct was analyzed in agarose gel. Retarded electrophoretic migration was observed for immune complex which depicts antibody binding to these modified polymers. These photoadducts also showed appreciable binding with anti-poly(dA-dT)-8MOP IgG in competition-inhibition experiment. These findings substantiate that anti-poly(dA-dT)-8MOP antibodies are recognizing the typical epitopes at the site of crosslinks, shared by poly(dA-dT)-8MOP, DNA-8MOP and DNA-psoralen photoadducts.

To probe the Z- or Z-like conformation at the site of photocrosslinking, anti-poly(dA-dT)-8MOP antibodies were incubated with DNA brominated in high salt (4 M NaCl) and used in competitive binding and gel retardation assay. The brominated DNA binding to anti-poly(dA-dT)-8MOP IgG was obvious by 51.3% inhibition in antibody-immunogen interaction. The antibody binding to brominated DNA was further confirmed by retarded electrophoretic migration of immune complex in agarose gel. The binding data reveals anti-poly(dA-dT)-8MOP antibody recognition of Z- or Z- like conformation; since native DNA brominated under high salt (4 M NaCl) has been shown to undergo Z- or Z- like transition.

The conformational distortion produced as a result of 8-MOP crosslinking between two strands of nucleic acid polymers was probed by monoclonal anti-ZDNA (Z22) antibody. Monoclonal Z22 IgG was found to be highly specific for prototype Z-DNA and at 10 ug/ml of Z-DNA concentration the Z22 IgG showed 78% inhibition. Furthermore, Z22-ZDNA interaction was significantly inhibited by poly(dA-dT)-8MOP and DNA-8MOP photoadducts. The Z22 recognition of photoadducts was also confirmed by retarded electrophoretic mobility of poly(dA-dT)-8MOP and DNA-8MOP complexed with Z22 IgG.

In conclusion, photoaddition of 8-MOP to DNA and poly(dA-dT).poly(dA-dT) gave rise to covalent crosslinked species that differ in various physico-chemical properties from unmodified polymers. Crosslink analysis suggested the involvement of thymine in photoadduct formation. The crosslinked photoadducts generated high titer immunogen specific antibodies which showed significant binding with Z-DNA. Of both photoadducts, the poly(dA-dT)-8MOP crosslinks were comparatively more immunogenic than DNA-8MOP crosslinks as revealed by direct binding and competition ELISA. The antibodies showed high specificity towards altered structure of immunogen as neither antibodies showed binding to A- or B- DNA. The conformational perturbations at the site of crosslink formation resulted in appearance of Z- or Z-like structure indicated by the binding data of monoclonal anti-ZDNA antibody.

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Prof. Rashid Ali (Supervisor)

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CERTIFICATE

I certify that the work presented in the following pages has been carried out by Miss. Zarina Arif and is suitable for the award of Ph.D. degree in Biochemistry of the Aligarh Muslim University, Aligarh.

(RASHID ALI)
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Dedicated
To
My Parents and
Brother
Dr. Feroz Ahmad

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As one steps out the first foot forward, perhaps, goal is the only attraction, but, then pleasure and pain, fulfilment and frustration, success and set-backs reveal the majestic enticing charm of journey, the life.

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CONTENTS

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ABBREVIATIONS

A_{260}	: Absorbance at 260 nm
BSA	: Bovine serum albumin
MBSA	: Methylated bovine serum albumin
DEAE	: Diethylaminoethyl
IgG	: Immunoglobulin G
High salt	: 4 M sodium chloride
ROS	: Reactive oxygen species
RT	: Room temperature
PUVA	: Psoralen plus ultraviolet-A light (nm)
uL	: Microliter
ug	: Microgram
Tm	: Mid-point melting temperature
dsDNA	: Double stranded DNA
nDNA	: Native DNA
ssDNA	: Single stranded DNA
8-MOP	: 8-Methoxypsoralen
5-MOP	: 5-Methoxypsoralen
TMP	: 4, 5', 8-Trimethylpsoralen
DMA	: 4', 5-Dimethylangelicin
AMT	: 4'-Aminomethyl-4,5',8-trimethylpsoralen
SLE	: Systemic lupus erythematosus
EDTA	: Ethylenediaminetetraacetic acid
TEMED	: N, N, N', N'- tetraethylmethylenediamine
Tris	: Tris-(hydroxymethyl) aminomethane
Br-DNA	: Brominated DNA
UV	: Ultraviolet
λex	: Excitation wavelength
λemit	: Emission wavelength

I INTRODUCTION

The discovery that genetic information is coded along the length of a polymeric molecule composed of only four types of monomeric units, is one of the major scientific achievements of this century. The polymeric molecule, DNA is the chemical basis of heredity and the "B form" is overwhelmingly dominant form of DNA under physiological conditions (high degree of hydration, low salt) (Saenger et al., 1986; Lu et al., 1992). Nothing else in human experience is remotely comparable to the compact packaging of large quantities of information in DNA, which is composed of two polynucleotide strands running in opposite direction that twist into a double helix so as to bring the bases into contact at the centre. The sugar phosphate backbone of the strands are exposed on the outside of the helix. About ten pairs of nucleotide residues, with their associated paired bases in the centre form one complete turn of the helix (Watson and Crick, 1953).

Biochemical investigations have brought penetrating new insight into the photochemical action of psoralens on biomolecules like DNA and RNA. The furocoumarins have been used extensively both as DNA modifying agents and as probes to analyze the structure and function of nucleic acids under variety of conditions (Cole et al., 1976; Ashwood-Smith et al., 1977; Shen et al., 1979; Bachellerie et al., 1981; Zolan et al., 1982; Saffran et al., 1988). Photochemotherapy with psoralen and long-wave ultraviolet light (UV-A) has been widely used for their antiproliferative activity in the treatment of various skin diseases (Stolk and Siddiqui, 1988; Takashima et al., 1991).

Structure and Types of Furocoumarins

Furocoumarins are wide spread umbelliferon derived bifunctional/monofunctional heterocyclic photoreagents which occur widely in nature (Pathak et al., 1961; Beier and Oertli, 1983; Cimino et al., 1985; Bisagni, 1992). These are skin photosensitizers which owe a remarkable photoinduced melanizing property and have proved active in the treatment ~~in the treatment~~ of vitiligo and of such skin diseases as psoriasis, eczema and mycosis fungoides (Ben-Hur and Song, 1984; Kang, 1992; Bisagni, 1992). A variety of furocoumarins are known till date. They include psoralen, 8-MOP, 5-MOP, 4,5′,8-trimethylpsoralen, angelicin, isohalofordin, tetrahydropsoralen, 4′-aminomethyltrioxsalen, 5-formyl-8methoxypsoralen, Khellin etc. Of these only 8-MOP, psoralen and 4,5′,8-trimethylpsoralen are of chemical importance as well as of therapeutic usefulness, photosensitivity and melanogenic properties (Hearst, 1981; Fujita and Kakishima, 1986).

Photobiophysical Properties of Psoralens

Furocoumarins in general and psoralen in particular are intercalating DNA binding drugs which have been widely studied because of their tremendous use in the photochemotherapy of skin diseases (Parrish et al., 1974, 1982; Fitzpatrick et al., 1982; Piette, 1992). The photobiological activity on UV-A (320-400 nm) irradiation relies on their photochemical reactivity with pyrimidines (Demaret and Brunie, 1990). Adduct formation is efficient in DNA because furocoumarins intercalate into the DNA helix and are in close association with the DNA bases (Specht et al., 1988; Lipson et al., 1988). The aromatic nature and molecular thickness of psoralen derivatives (5-MOP or 8-MOP) are suitable to intercalate into the space between stacked base pairs in DNA where the hydrophobicity is moderate (Sasaki et al., 1987). Adduct formation with nucleic acids takes place in three

successive steps namely; molecular complexing, photobinding of furocoumarin to DNA through bases and finally crosslinking of furocoumarin between the strands of DNA (Cimino et al., 1985; Kim et al., 1988). Furocoumarins have low solubility in water but in the presence of nucleic acids their solubility increases. They form a weak molecular complex in nucleic acid solution which involves weak bonding forces such as hydrogen bonding, Van der Waals and hydrophilic forces. Upon UV irradiation (320-400 nm) intercalated psoralens can photoreact with adjacent pyrimidine bases mostly with thymidines in DNA (Straub et al., 1981; Kanne et al., 1982b; Calvin and Hanawalt, 1984; Van Houten et al., 1986; Pfluger and Ostrander, 1989; Gia et al., 1992) and uridines in RNA (Pfluger and Ostrander, 1989), to form first monoadduct which are linked to only one strand of the helix and then diadducts which are linked to both strands of the helix (Song and Tapley, 1979; Parsons, 1980; Cimino et al., 1985). In the first step of photoreaction either a furan side or a pyrone side monoadduct is formed depending upon whether the 4', 5' double bond or 3,4 double bond of the psoralen reacts with the 5, 6 double bond of a pyrimidine base (Fig. 1, Step B). By absorbing a second photon, the furan side monoadduct can be converted to diadduct (interstrand crosslink) if there is a pyrimidine base available for photoreaction on the other strand (Tessman et al., 1985; Shi and Hearst, 1987 a). In contrast, the pyrone side monoadduct can not be driven to a diadduct because it does not absorb photons in the 320-380 nm wavelength region (Shi et al., 1988 a, b). All these adducts are photoreversible upon exposure to light in their respective absorption regions (Cimino et al., 1986; Shi and Hearst, 1987 b). Table 1 refers to what is known at present about the various photophysical and photochemical events that can occur from an excited psoralen molecule.

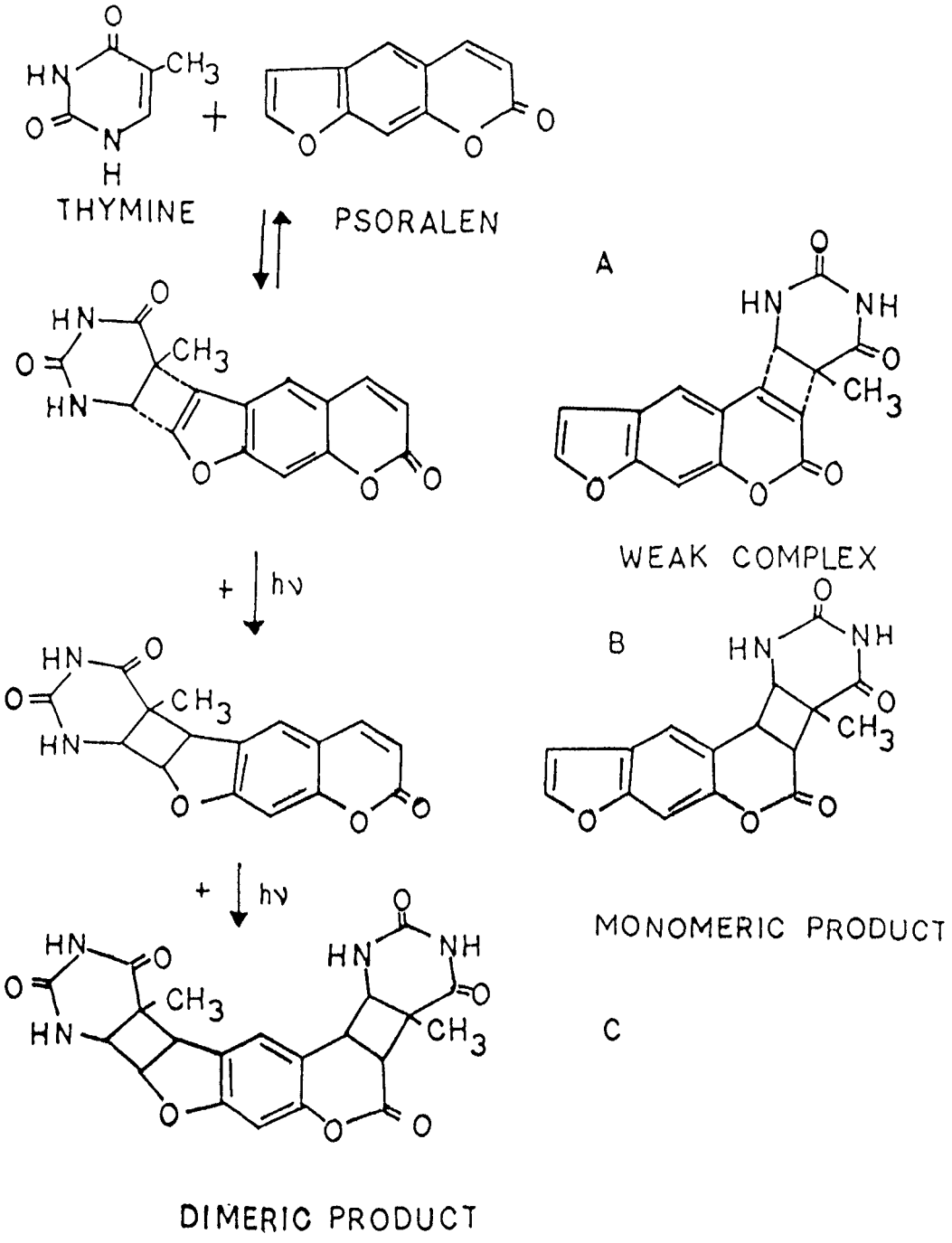

Fig.1. Schematic representation of steps involved in psoralen photosensitization.

TABLE 1

Photophysical and photochemical events following UV-A irradiation of psoralens*

EFFECTS	
1. Fluorescence and phosphorescence emission	5. **Photoadditions**
2. Dimerization	a) Pyrimidine bases of DNA, RNA
3. Energy transfer: singlet oxygen generation	b) Flavin-mononucleotide
4. Free radical formation	c) Amino acids of proteins
	6. **Oxidation**
	a) amino acids
	b) unsaturated fatty acids
	d) Membrane components

*Source: Rodighiero, G. and Dall'Acqua, F. (1986) Drugs Expt. Clin. Res. 12, 507-515.

Monoadducts and crosslinks lead to important genotoxic effects when induced in living organisms. Like most physical and chemical carcinogens interacting primarily with DNA, psoralen adducts are mutagenic (Bridges et al., 1979; Averbeck, 1985, 1989). It appears that mutations owing to psoralens are targeted and that transversion, frame shift events and deletions are induced (Piette, 1992). Psoralens are also phototoxic, mutagenic and carcinogenic for eukaryotic cells like yeast (Averbeck and Moustacchi, 1979) and mammalian cells (Papadopoulo and Averbeck, 1985; Papadopoulo et al., 1986; Young, 1986). It is generally thought that psoralen DNA diadducts are potentially more deleterious as they cause cell killing and mutagenesis, whereas monoadducts are more easily repaired (Averbeck and Moustacchi, 1980; Wani and Arezina, 1991; Munn and Rupp, 1991). However, because a crosslink damages bases at nearly the same position in both strands, it cannot be repaired by simple excision mechanism or by any scheme that requires retention of complementary base pairing information at that dinucleotide position (Calvin and Hanawalt, 1987). The presence of a crosslink prevents strand separation and should therefore constitute a complete block to replication (Calvin and Hanawalt, 1987). Previously it was indicated that psoralen photoinduced crosslink is biologically the most relevant DNA lesion (Ben-Hur and Elkind, 1973). However, more recent investigations using mono- and bifunctional furocoumarins plus UV-A (Averbeck et al., 1975; Bordin et al., 1976; Averbeck, 1985; Papadopoulo and Averbeck, 1985) or bifunctional furocoumarins in combination with irradiation at different wavelengths (Averbeck et al., 1987; 1990a; Cundari and Averbeck, 1988; Papadopoulo et al., 1988) highlighted the importance of the simultaneous presence of 2 types of lesions (monoadduct and crosslink) probably involving processing by different repair pathways (Moustacchi et al., 1983; Averbeck et al., 1990 b). It is

unclear whether the presence of one type of lesion affects the repair fidelity and/or efficiency of the other type. There is an increase in non specific DNA incision when the ratio of 8MOP-UV-A induced crosslink to monoadduct is increased (Cundari et al., 1991). Comparison of the mutagenic effects caused by monoadducts and crosslinks have been made and the mutagenic efficiency, in various E. coli strains was found to be TMP> 5-MOP> 8-MOP> psoralen> angelicin> 4,5'-dimethylangelicin (Averbeck et al., 1990 b).

The identification of reactive state of furocoumarins which give rise to mono- and diadduct is a topic of debate and has intrigued the photobiologists for several years. The spectroscopical studies show that psoralen and their derivatives have two transitions in the 320-400 nm range; an n - π^* transition resulting from the excitation of the non bonding electron on the C-2 carbonyl group to the π^*orbital, and a π - π^* transition occurring when a π electron in the psoralen ring system is excited to the π^* orbital. Lowest singlet states (Mantulin and Song, 1973) and triplet states (Mantulin and Song, 1973) are the (π, π^*) states. The reactivity of either 1(π, P^*) or the 3 (π, π^*) towards pyrimidine cycloaddition is determined by kinetics (i.e. life time), steric and electronic factors. The triplet excited state which is localized in the 3,4-carbon-carbon double bond was predicted to be more reactive than the singlet state (Song et al., 1971). The active participation of the triplet state of free furocoumarin in solution has been well established and confirmed by observing dynamic triplet quenching of the photoreaction process upon addition of nucleic acid bases and amino acids (Bensasson et al., 1978; Beaumont et al., 1979).

It has been suggested that the 4',5' photomonoadducts are formed via a singlet excited state

of psoralen upon absorption of the first photon, followed by crosslink (diadduct) formation via the triplet state upon absorption of a second photon (Beaumont et al., 1979) although this has not been established. However, the triplet excited states of 4', 5' dihydropsoralen (Land and Truscott, 1979) and the 4', 5' photoadduct of psoralen and thymine (Bensasson et al., 1980) are both quenched by thymine.

The electron density in the reactive regions of the furocoumarin is localized in the 3,4 or 4', 5' double bond positions, get disturbed due to intramolecular charge transfer from the π - electron system to 3, 4 or 4', 5' double bond. Theoretical calculation of the electron density in these excited states show that 3,4 double bond has more charge transfer character than the 4', 5' double bond (Mantulin and Song, 1973; Song, 1984). Based on these results it has been suggested that the triplet photoreactivity resides with pyrone moiety rather than furyl group (Mantulin and Song, 1973; Song, 1984). Reactivity of the 4', 5' double bond therefore appears to be determined by steric and kinetic factors rather than electronic factors.

Photoreactivity of Nucleic Acids with Furocoumarins

The photochemotherapeutic potency of various furocoumarins depends on its photobinding capacity to nucleic acids (Fujita and Kakishima, 1986; Shoonderwoerd et al., 1991). Thymidine appears to be the primary target for psoralen addition (Straub et al., 1981; Kanne et al., 1982 a, b; Sastry et al., 1992) but a very minor reaction also occurs with cytosine in DNA (Turner and Noller, 1983; Garrett-Wheeler et al., 1984; Sastry et al., 1992). Strong sequence effect on the formation of photoadduct has been observed, repeated A-T sequences are "hot spot" for psoralen photobinding (Sage and Moustacchi, 1987; Bredberg and Nachmansson, 1987; Sage and Bredberg, 1991).

However, 5'-TpA sequence is favored over the 5-ApT sequence (Gamper et al., 1984; Zhen et al., 1986; Sage and Moustacchi, 1987) recent investigations have pointed out modulating effects of flanking sequences (Gia et al., 1992).

The poly(dA-dT).poly(dA-dT) sequence region is apparently the most favorable site for both intercalation and photocycloaddition of furocoumarin (Kang, 1992). Poly(G-C) sequences are not favorable for photocycloaddition although intercalation in the G-C sequences are not inhibited (Kang, 1992). The polynucleotide regions corresponding to the sequence of poly(dA).poly(dT) or poly(dG).poly(dC) are apparently not suited for optimal intercalation and subsequent photocyclo-addition (Dall'Acqua et al., 1979 a).

Presently the role of adenine base in the photocyclo-additivity of furocoumarins towards thymine bases in DNA cannot be explained in detail, but adenine definitely plays an important role in the photoreactivity or photoreaction path, since a longlived transient species is also generated as a primary product of quenching of coumarin excited singlet states by bases Kang in 1992 proposed that adenine base mediates an electron transfer from furocoumarins to thymine base resulting in cycloaddition of furocoumarins to thymine:

$$^{1}FC + A \longrightarrow FC^{\cdot -} + A^{\cdot +}$$
$$FC^{\cdot -} + T \longrightarrow FC^{-} - T^{\cdot}$$
$$FC^{-} - T^{\cdot} + A^{\cdot +} \longrightarrow FC <> T + A$$

where FC is furocoumarin, A and T are adenine and thymine bases respectively. This proposal implies that purine bases, especially adenine play an important role in photocycloaddition of furocoumarins to pyrimidine bases especially thymine in DNA and consequently suggests that

termolecular interaction of adenine-furocoumarin-thymine significantly contributes to photocyclo-addition of excited singlet furocoumarins to thymine base in DNA.

As the presence of different bases in polynucleotides effect its photoreactivity towards furocoumarins, the substituents of a psoralen effect each step in its interaction with nucleic acids (Cimino et al., 1985). The ability to dark bind and photoreact with DNA and RNA depends on the position, steric and the electronic characteristics of each group on the psoralen ring system. The photochemistry of a methylated psoralen and isopsoralen is relatively fast. Methylation of a psoralen and isopsoralen increases the dark binding affinity, the quantum yield of photoaddition, and the quantum yield of photobreakdown of the compound (Guiotto et al., 1981; Isaacs et al., 1982; Dall'Acqua et al., 1983). 4,5', 8-trimethylpsoralen and 4,4', 6-trimethyl-psoralen are therefore much more reactive than unsubstituted psoralen or isopsoralen respectively (Guiotto et al., 1984). A methoxy group at 8 position slows the photochemistry, with 8-methoxypsoralen adding much more slowly to DNA, but being much longer lived in solution than 4,5', 8-trimethylpsoralen (Isaacs et al., 1982). Benzopsoralens and benzoangelicin are photochemically monofunctional reagents. The introduction of benzene ring at the level of furan side of 4,6-dimethylangelicin increases the affinity of the new compound towards DNA in terms of non covalent complex formation (Vedaldi et al., 1992).

It is a well established fact that only the linearized type of furocoumarins possess the potentiality of crosslink formation whereas angular forms are devoid of this property (Vigny et al., 1987). Various attempts have been made to establish the relative mutagenicity, carcinogenicity of monoadducts versus diadducts or crosslinks and the clinical application of monoadduct in

the photochemotherapy (Gange et al., 1984). Also, the different kinds of cellular response is elucidated against the two types of adducts.

Angelicin having angular structure forms only monoadducts. It has unique property of having misaligned two reactive double bonds once it has formed monoadduct, then subsequent reaction of the remaining double bond with pyrimidine in the adjacent nucleic acid strand to form a crosslink is completely ruled out (Young, 1990). Inspite of its unique property it has been reported to form crosslinked adduct with phage lambda DNA (Lown and Sim, 1978; Kittler et al., 1980; Kittler and Lober, 1983). This may be due to the special folded structure within the phage head, which may be ultimately responsible for the formation of crosslink. Some of the properties of mono- and bifunctional psoralens are depicted in Table 2.

Photoreactions of Furocoumarins with Proteins, Biomembranes and Lipids

The photoexcited furocoumarin derivatives are able to react efficiently with proteins (Dall'Acqua and Caffieri, 1988; Midden, 1988; Beijersbergen Van Henegouwen et al., 1989; Frederiksen et al., 1989; Labbe et al., 1989; Dall'Acqua and Martelli, 1991; Kumar et al., 1992). The furocoumarin-protein interaction during UV-A irradiation has demonstrated that not only 8-MOP (Yoshikawa et al., 1979; Lerman et al., 1980) but also other linear (psoralens) and angular (angelicin) furocoumarins (Veronese et al., 1981) photobind to proteins as they do to nucleic acids (Musago and Rodighiero, 1972). Also, its photoreaction with enzyme causes a change in its catalytic activity (Talib, 1975; Mitra et al., 1984). PUVA therapy induces various types of damage to proteins (Meffert et al., 1977; Bertaux et al., 1981; Schiavon and Veronese, 1984, 1986; Schiavon et

TABLE 2

Some properties of mono- and bifunctional furocoumarins*

Property	Bifunctional (crosslinking)	Monofunctional
1. Molecular structure	Mostly linear	Mostly non-linear. Exception 3-carbethoxypsoralen
2. Skin photosensitizing activity	Strongly photo-toxic	Absent nonerythemogenic
3. Ability to form		
singlet oxygen	Strong	Moderate to strong
molecular oxygen	Moderate	Moderate to strong
free radical	Strong	Weak to strong
4. Mutagenicity	Strong	Moderate to strong
5. Stimulation of melanin pigmentation	Strong	Weak
6. Carcinogenicity	Topical-strong	Topical-moderate to strong
	Oral-weak	Oral-not investigated
7. DNA interaction	Strong	Weak to moderate
8. Photobinding capacity to DNA	Strong	Weak to moderate
9. Monoadduct forming capacity with DNA	Strong	Strong
10. Interstrand cross-linking with DNA	Strong	Absent in dilute aqueous solutions
11. Inhibition of DNA and RNA synthesis	Strong	Moderate to strong
12. Therapeutic effectiveness in psoriasis	Strong-well investigated	Weak to moderate-not extensively investigated
13. Therapeutic effectiveness in other diseases (vitiligo, mycosis fungoides)	Strong and effective	Not investigated

*Source: Kittler, L. and Lober, G. (1988) Stud. Biophys. 124, 97-114.

al., 1984; Malinin et al., 1986; Vedaldi et al., 1987). Furocoumarins possess unique property of sensitizing the photopolymerization of subunits of oligomeric proteins (Schiavon and Veronese, 1984) but exert no effect on monomeric protein (Schiavon and Veronese, 1984). The sensitized photolysis of some amino acids including tryptophan, methionine, tyrosine and histidine has also been observed (Veronese et al., 1982).

Furocoumarins are known to interact at specific sites with biomembranes both in dark and after UV-A irradiation (Potapenko, 1991). There are several types of highly specific binding sites of furocoumarins on the surface, in man and mice cells (Laskin et al., 1985). These sites are covalently modified by psoralens after UV-A irradiation, with significant photobiological effects. For instance, the binding of epidermal growth factor with the corresponding receptor is inhibited (Laskin et al., 1986), because of inhibition of tyrosine kinase activity of the epidermal growth factor receptors (Mermelstein et al., 1989). PUVA also causes an increase in the permeability of leukocytes (Bohm et al., 1986) and erythrocyte membrane (Potapenko et al., 1986 a, b, 1988, 1991; Lysenko et al., 1988).

Psoralens can also form photochemical adducts with unsaturated fatty acids (Cadet, 1990) and membrane lipids (Schoonderwoerd et al., 1991). PUVA modification of unsaturated lipids occur by two pathways namely: oxygen dependent (photodynamic) and oxygen independent (Potanpenko, 1991).

Ultraviolet irradiation of TMP or 8-MOP with free unsaturated fatty acids or methyl esters (oleic, linoleic, linolenic, or arachidonic acid) in methanol-water solution caused the formation of photoproducts (Kittler and Lober, 1984; Kittler et al., 1986 a, b). No

such photoproducts were observed with completely saturated fatty acids like stearic acid (Cadet, 1990). Specht et al. (1988) demonstrated that furocoumarins illuminated with near ultraviolet light form covalent adducts with unsaturated fatty acids and the rate of this reaction is comparable to the rate of formation of furocoumarin adducts with nucleic acid. Addition of furocoumarin is observed to occur at 3,4 bond of the coumarin portion of TMP (Specht et al., 1989) and addition of psoralens occurs at the 4', 5' double bond of the furan ring (Caffieri et al., 1988). This is in contrast with the reaction observed with pyrimidines in double stranded DNA, with this substrate the 4', 5' bond of the TMP appears to be the first to react (Kanne et al., 1982 b). This difference may be due to difference in relative orientation of the psoralens and substrate. Adduct formation is faster in polar solvents such as ethanol than in non polar solvents such as benzene (Cadet, 1990).

It has been hypothesized that psoralen-lipid adduct formation may play an important role in the therapeutic benefit of PUVA therapy (Midden, 1988; Midden and Klaunig, 1988). If this hypothesis is confirmed it will open a new pathway for the improvement of the safety of PUVA therapy for psoriasis by suggesting a new and straight forward strategy for eliminating the carcinogenicity of this treatment. More research is certainly warranted to determine the role of psoralen-lipid, psoralen-protein and psoralen-nucleic acid adducts in the biological effects of these intriguing drugs (Cadet, 1990).

Structural Deformation of the DNA Helix by Furocoumarins

It has been well documented that psoralens have preference for photobinding and crosslinking A-T rich compared to G-C rich DNA sequences (Chandra et al., 1973;

Dall'Acqua et al., 1978; Lown and Sim, 1978; Kanne et al., 1982 a, b). The relative initial rates of 4,5',8-trimethylpsoralen photobinding to poly(dG).poly(dC), poly(dG-dC).poly(dG-dC), poly(dA).poly(dT), poly(dA-dC).poly(dG-dT), calf thymus DNA and poly(dA-dT).poly(dA-dT) was calculated to be approximately 1, 2, 3, 5, 16, 30 and 150 respectively (Sinden and Hagerman, 1984).

Various forms of DNA like A-DNA, B-DNA, C-DNA, H-DNA, V-DNA and Z-DNA are known to exist. Out of these forms B-type helix (B-DNA possessing a base pair orientation perpendicular to the helix axis) appears to be the most suitable conformation for crosslinking (Lober et al., 1982; Kittler and Lober, 1983, 1988). The homopolymer poly(dA).poly(dT) is known to differ from classical B-DNA. By contrast, alternating heteropolymer poly(dA-dT).poly(dA-dT) is more like normal "B-DNA" and is capable of winding around nucleosome core (Yuan et al., 1992). Every base pair in poly(dA-dT).poly(dA-dT) is a potential crosslinking site for 8-MOP/furocoumarin (Gasparro and Santella, 1988). The photobinding of 4,5',8-trimethylpsoralen to Z-DNA is greatly reduced relative to DNA in B- conformation (Sinden and Kochel, 1987). In addition, its photobinding is strongly enhanced at B-Z junctions (Kochel and Sinden, 1989).

The effect of adduct on the local structure of the DNA depends on the psoralen derivatives. A conformational change is induced in the nucleic acid when it photoreacts with 8-MOP. Typically, 8-MOP photoadducts provoke an unwinding of 28^{o} in the double helix (Wiesehahn and Hearst, 1978). The X-ray crystallographic analysis of 8-MOP-thymine monoadduct have suggested that psoralen crosslinks will bend DNA, perhaps as much as 70^{o} (Peckler et al., 1982; Kim et al., 1983). Pearlman et al. (1985) have emphasized a strongly favored psoralen photobinding with sequences able to form kink, since they

have suggested that psoralen crosslinks will bend DNA. On the basis of electrophoretic migration of psoralen crosslinks in DNA, Sinden and Hagerman (1984) concluded that the psoralen crosslinks do not produce significant bends in the DNA helix, in contrast to the prediction from the X-ray crystallographic analysis of psoralen-thymine monoadduct. The intercalation and subsequent photobinding of psoralen to DNA may only unwind the DNA and results in an increase in length approximately equivalent to a base pair per photobound psoralen molecule. It is possible that a slight bending or other distortion of the DNA at the crosslink site (or even at monoadduct) exists, but it is not detectable using polyacrylamide gel electrophoresis (Zhen et al., 1988). The failure to observe crosslink associated 70^{o} bends in duplex DNA undoubtedly reflects the constraints placed upon the psoralen crosslink geometry by the helix structure itself (Sinden and Hagerman, 1984). However, the two dimensional proton NMR data of double stranded deoxyoligonucleotide containing a 4'-aminomethyl-4,5',8-trimethylpsoralen-thymine diadduct shows that the diadduct induces a localized kink and unwinds the DNA helix by about 56^{o} (Tomic et al., 1987) and this kink was predicted to be greater than that caused by a pyrimidine dimer (Pearlman et al., 1985). Moreover, Haran and Crothers (1988) observed a 10 degree of DNA bending by psoralen crosslinking and unwinding by about 1 bp, as well as stiffening of the double helix. A possible explanation for these controversial results could be that the DNA helix around a psoralen diadduct is flexible. Therefore, the kinking effect of a psoralen diadduct cannot be detected by dynamic methods such as gel electrophoresis or decay of linear birefringence (Shi et al., 1988 b)

Photobiological Effects of Nucleic Acid-Furocoumarin Interactions

The photoreactivity of psoralens with nucleic acids have found immense applications in medical and biological fields. Psoralens are used for probing nucleic acid structure (Song and Tapley, 1979; Parsons, 1980; Calvet et al., 1982; Setyono and Pederson, 1984; Cimino et al., 1985; Rinke et al., 1985; Shim et al., 1990), nucleic acid-protein interaction (Schwartz et al., 1983). Psoralen molecules provide a valuable tool for the study of *in vivo* structures, because they readily penetrate intact cells and viruses without disruption of membranes and viral capsids and can bind preferentially to the linker regions of nucleosomal DNA (Ostrander et al., 1986). This preferential binding has led to their wide use as probes of chromatin structure (Hanson et al., 1976; Wiesehahn et al., 1977; Cech and Pardue, 1977; Hallick et al., 1978). Furocoumarins have been widely used as a valuable tool to locate conformational variations and the secondary structure of *E. coli* 16S RNA (Wollenzien et al., 1978), *E. coli* 5S RNA (Rabin and Crothers, 1979) and *E. coli* 23S RNA in solution (Turner and Noller, 1983). The nucleic acid psoralen photoreaction has been widely used in the fixation and stabilization of variety of nucleic acid structures including transcriptional complexes, replicative intermediates, R-loops and D-loops (Cimino et al., 1985).

Human diseases have been treated with sun rays (Harber and Bickers, 1989; Epstein, 1990) since antiquity. However, photochemotherapy with psoralens and high intensity UV-A radiation was found effective in the treatment of severe psoriasis, mycosis fungoides and over 16 other skin diseases (Scott et al., 1976; Makki et al., 1991; Averbeck et al., 1992; Cadet et al., 1992; Gia et al., 1992; Pathak and Fitzpatrick, 1992). Psoralens are now emerging as a photoprotective agents against non

melanoma skin cancers and as immunologic modifiers in the management of patients with disorders of circulating T-cells using new techniques of photopheresis (Pathak and Fitzpatrick, 1992), as well as enhanced tanning of skin which might protect from the harmful effects of sun light (Pathak, 1982).

The effect of PUVA in the treatment of psoriasis appear to result from a direct inhibition of DNA synthesis and mitosis in the hyperproliferative epidermal cells (Epstein et al., 1970; Ronto et al., 1992) and also the inactivation of enzyme system (proteins) which contributes to the occurrence of therapeutic effect (Beijersbergen Van Henegouwen et al., 1989). PUVA induced immunosuppression is thought to be related to a reduction in the number of antigen presenting Langerhans cells (Stingl et al., 1986; Ashworth et al., 1989). 8-MOP plus UV light can suppress contact hypersensitivity in mouse (Kripke et al., 1983), alter the proportion of circulating lymphocytes (Morison et al., 1981) and inhibit mitogen and alloantigen induced lymphocyte proliferation (Friedmann and Rogers, 1980; Morhenn et al., 1980; Bredberg and Forsgren, 1984; Gasparro et al., 1984; Berger et al., 1985). PUVA therapy induces point/gene mutations and chromosome aberrations in a variety of organisms (Scott et al., 1976). PUVA therapy also has a long term side effect of premature aging of the skin (Oikarinen et al., 1985), presumably due to the production of reactive oxygen species (Carbonare and Pathak, 1992). Some of the photobiophysical properties of furocoumarins are presented in Table 3.

There are serious concern about PUVA therapy that might induce SLE like syndrome (Kubba et al., 1980). The therapy may have been precipitating factor in the development of SLE like syndrome (Millins et al., 1978). It is also possible that PUVA therapy merely contributed

TABLE 3

Photobiophysical properties of furocoumarins*

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1. Induction of erythema and blisters on skin
2. Increase of the dark pigmentation of skin
3. Photoaging of the skin
4. Killing of bacteria
5. Inhibition of viruses and phages
6. Inhibition of the capacity of tumor cells to transmit the tumor
7. Inhibition of DNA, RNA and protein synthesis inside living cells
8. Inactivation of enzymes
9. Induction of mutants in both bacterial and mammalian cells

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*Source: Rodighiero, G. and Dall'Acqua, F. (1986) Drugs. Expt. Clin. Res. 12, 507-575.

to the risk factor in patients who were otherwise predisposed to ANA developing (Kubba et al., 1980). Native DNA is a weak antigen, but exposure of DNA to UV radiation or to psoralen and UV-A light alters the native structure of the molecule so that it becomes immunogenic (Zarelska et al., 1978). The antibodies are highly specific and they do not cross react (Levine et al., 1966; Tan, 1968; Eyanson, 1979). However, because of theoretical concern for systemic immunologic effects that may be associated with long term PUVA therapy and may induce the production of ANAs over many years or precipitate autoimmune disease as an idiosyncratic effect, continued monitoring is required (Stern et al., 1979).

Systemic lupus erythematosus is an autoimmune disease of unknown etiology. Autoimmune diseases are those which result from immunologic reactions, humoral and/or cellular, directed against the individuals own tissue components. Recently many clinical diseases have been assigned an autoimmune origin, but the evidence may not be equally convincing for all. It is abundantly clear that no single theory or mechanism can adequately explain all features of autoimmune diseases (Deodhar, 1992). The best approach as suggested by Shoenfeld and Isenberg (1989) is to consider the wide spectrum of autoimmune diseases as the mosaic of autoimmunity with its many pieces, genetic, hormonal, immunological and environmental leading to diverse diseases. The old "forbidden clone theory" has undergone several modifications, and more recently has essentially been abandoned in favour of the multifactorial considerations which help to explain not only the diversity of various clinical diseases encountered, but also the differences in clinical manifestations from patient to patient with the same disease group. Table 4 represents some autoimmune diseases and their respective autoantibodies.

TABLE 4

Diagnostic antibodies associated with various autoimmune disorders*

Disease	Autoantibodies directed against
A. *SLE and Related Diseases*	
Systemic lupus erythematosus	Nuclear antigens, DNA, Sm, cardiolipin
Scleroderma (PSS)	Nuclear antigens, Scl-1, Scl-70
Sjogrens' syndrome	Nuclear antigens, SS-A, SS-B
Mixed connective tissue disease	Extractable nuclear antigen (RNP)
Rheumatoid arthritis	Fc portion of IgG (rheumatoid factor), nuclear antigens
B. Other Autoimmune Diseases	
Autoimmune thyroid diseases	Thyroglobulin, microsomal Ag, TSI
Addison's disease	Adrenal cortical cell Ag
Pernicious anemia	Intrinsic factor, parietal cell Ag
Inflammatory bowel diseases	Colonic mucosal Ag
Chronic active hepatitis	Smooth muscle Ag
Primary biliary cirrhosis	Mitochondrial Ag
Myasthenia gravis	Acetylcholine receptor
Goodpastures syndrome	Glomerular basement membrane
Pemphigus vulgaris	Epidermal intercellular substance
Bullous pemphigoid	Skin basement membrane
Autoimmune hemolytic anemia	Red cell membrane Ags
Multiple sclerosis and related demyelinating diseases	Myelin basic protein Ag
Diabetes type I	Islet cell Ag (also cytotoxic T cells)
Wegener's granulomatosis and other vasculitides	Serine protease and myeloperoxidase (C-ANCA, P-ANCA)

*Source : Deodhar, S.D. (1992) Clin. Biochem. 25, 181-185.

Immunogenicity of Nucleic Acids

Chemically or physically modified DNA or helical structures that differ significantly from the "B-helix" are much stronger immunogenic stimuli than native DNA and most of the antibodies induced by modified DNA do not react with unmodified conformer (Stollar, 1986, 1989). Depending on the mode and type of modification, it may valuably contribute to the phenomenon of carcinogenicity, mutagenicity and genotoxicity in living systems.

a) **Antibodies to nucleic acid modified by furocoumarin photoaddition**

Alteration in the nonimmunogenic native DNA conformation by 8-MOP photoaddition yields highly immunogenic conformer. Recently it has become possible to quantitate specific modifications on DNA by immunological methods. A panel of antibodies have been developed which specifically recognize the DNA adducts of several carcinogens including benzo [a] pyrene (Santella et al., 1984), aflatoxin (Haugen et al., 1981; Herstzog et al., 1982) and ethyl nitrosurea (Rajewsky et al., 1980) as well as thymidine dimers (Strickland and Boyle, 1981) and thymine glycol (Leadon and Hanawalt, 1983) produced in DNA exposed to ionizing or UV radiation. These antibodies are used as valuable reagents to detect or quantitate adduct levels in various animal and human samples. Monoclonal antibodies against DNA-8MOP have been used to monitor adducts in psoriasis and cancer patients treated with psoralen plus UV-A light (Santella et al., 1990).

A panel of monoclonal antibodies have been developed which specifically recognize DNA modified by 8-MOP and UV-A light (320-400 nm) (Santella et al., 1985). These antibodies were highly specific for the modified DNA and showed no cross reactivity with the unmodified conformer. However, the polyfunctional nature of psoralens

complicates the determination of the specificities of the monoclonal antibodies. The induced antibodies revealed the formation of monoadducts and diadducts in the synthetic polymer poly(dA-dT).poly(dA-dT). However, the antibodies showed the formation of only monoadducts in the double helical structure of poly(dA).poly(dT). This was due to the absence of thymines on the opposite strand of the polymer (Santella et al., 1985). The monoclonal antibodies against drug photoadded to poly(dA).poly(dT) showed a strong binding potential with the immunogen. The binding results suggest that the monoclonal antibodies were recognizing the 8-MOP monoadduct regions. Surprisingly, these antibodies had a six fold lower sensitivity towards the monoadduct rich samples of poly(dA-dT).poly(dA-dT) (Santella et al., 1985).

All the monoclonal antibodies developed have some cross reactivity with AMT and DMA modified DNA. Since 8-MOP and AMT differs only in the methyl and methoxy substitution around the ring, cross reactivity would be expected. Angelicin, however, differ in its ring structure being an angular psoralen derivative capable of forming only monoadducts (Song and Tapley, 1979). Its reactivity indicates that the antibodies recognize structures that are present on the coumarin ring. Furthermore, the antibody might cross react with crosslinked adducts, it probably does not specifically recognize the cyclobutyl moieties.

Recently, polyclonal antibodies against DNA-psoralen crosslinks have been generated (Hasan et al., 1991). The induced antibodies were highly specific for the crosslink. Surprisingly the antiserum showed appreciable binding with the left handed Z-DNA as well as brominated DNA. The recognition of Z-DNA by anti-crosslink antibodies is suggestive for the presence of Z-or Z-like structure on DNA-psoralen photocrosslink.

b) Antibodies against left handed Z-DNA

The dynamic DNA structure can exist in various conformational states influenced by its microenvironment and base pair sequence. As a consequence it undergoes into conformation of new helical geometries that are more varied and are potentially immunogenic (Stollar, 1975; Lafer et al., 1981; Saenger, 1984; Laprete and Hartman, 1989). Of these conformationally altered polymers Z-DNA has received much attention because of its possible involvement in the control of gene expression (Rich et al., 1984; Stollar, 1986). Thus in view of its role in gene regulation, specific antibodies against the conformer has been generated to locate the possible occurrence of such type of structures in natural DNA.

Both polyclonal and monoclonal antibodies have been induced with several forms of Z-DNA (Stollar, 1986). Some population of the induced antibodies exhibited selectivity for the particular base composition and sequence used for immunization, whereas others are more general anti-ZDNA antibodies (Zarling et al., 1984; Nordheim et al., 1986). Polyclonal sera induced by brominated poly(dG-dC) are of the latter kind, reacting with the immunogen and also with poly(dG-dm5C), poly(dG-dBrC), or mixed base sequences having Z-DNA structure (Lafer et al., 1983). However, monoclonal antibodies from mice immunized with brominated poly(dG-dC) showed different kind of reactivity. One monoclonal antibody, Z22, reacts with Z-DNA of variable sequence, whereas another, Z44, is selective for Z-DNA formed by poly(dG-dC) sequences; it does not react, for example, with poly(dG-dm5C) or poly(dT-dG).poly(dC-dA) or other mixed base sequences in the Z-helical form (Nordheim et al., 1986).

c) Immunogenicity of triple helical H-DNA

H-DNA (triple helical) is a conformational variant of the genetic material and found to be highly immunogenic in experimental animals. An H-DNA can be constructed by taking a mixture of poly(dT-dm5C) and poly(dG-dA) which can form a three stranded helix at neutral pH, with two of the poly(pyrimidine) strands flanking a central poly(purine) (Lee et al., 1980). The two poly(pyrimidines) have same polarity, but in one of them the cytosine is protonated. Monoclonal antibodies have been raised against H-DNA, that reacts specifically with this complex polymer and fails to react with related polynucleotide that cannot form a triple helix (Lee et al., 1987). For example, these antibodies do not react with a mixture of poly(purine) poly(dG-dm6A) and the poly(pyrimidine) poly(dT-dm5C), because a triple helix can not be formed here due to the inability of the 6-methyladenine to form hydrogen bond with two pyrimidines. Furthermore, the monoclonal anti-triplex antibody does not react with native mammalian or bacterial DNA. However, these antibodies gave positive immunofluorescence test with metaphase chromosomes and interphase nuclei, thereby providing ample evidence for the occurrence of triple helical DNA in these complex structures (Lee et al., 1987).

d) Antibodies against poly(dG).poly(dC)

Poly(dG).poly(dC) is the only unmodified right handed duplex DNA that is strongly immunogenic as it deviates slightly from average B-DNA. They elicit specific antibodies that recognize the difference between them and native B-DNA. Polyclonal antiserum to poly(dG).poly(dC) revealed the presence of two population of antibodies: One population directed against the immunogen and one minor population directed against poly(dG) (Stollar, 1989). However, this antiserum did not recognize native calf or bacterial DNA (Lafer and

Stollar, 1984). Monoclonal antibodies specific for poly(dG).poly(dC) also distinguished this polymer from native DNA (Lee et al., 1984).

e) Antibodies to poly(dA).poly(dT)

The double helical homopolymer poly(dA).poly(dT) have been found to induce specific antibodies in experimental animals (Diekmann and Zarling, 1987). The elicited antibodies were unreactive towards native DNA and other synthetic polynucleotides. However, it reacted specifically with the protozoal kinetoplast DNA regions which contain sequences of oligo(dA) on one strand and oligo(dT) on the other. Similarly, experimental animals challenged with Leishmania kinetoplast DNA were found to elicit antibodies with the same specificity. The antibodies induced against poly(dA).poly(dT) as well as kinetoplast yielded immunofluorescent staining of both nuclei and kinetoplasts in situ. The staining was inhibited by soluble poly(dA).poly(dT) but not by purely B-DNA polymers. These findings reflect the differences in structure between poly(dA).poly(dT) and B-helical DNA (Arnott et al., 1983) and the presence of varying configurations within naturally occurring DNA. The repetition of an oligo(dA).oligo(dT) sequence at appropriate spacing can give rise to a bend in DNA helix (Marini et al., 1982; Trifonov, 1985; Koo et al., 1986). It may be this kind of feature that is recognized by the antibodies.

f) Antibodies to poly(dT-dC).poly(dG-dA)

Monoclonal antibodies to poly(dT-dC).poly(dG-dA) was prepared by Lee and coworkers in 1985. These antibodies were found to be polyspecific in character. The antibody bound to several polypeptide immobilized on a solid phase, but showed greater selectivity in binding to competing nucleic acids in solution. Significant reactivity was retained by a polymer in which dU replaced

dT and a seven fold enhancement of binding (heteroclitic) was caused when dBr5C replaced T. However, native calf thymus DNA was a weak competitor with a relative affinity just one hundredth to that of the immunogen. Thus on the basis of its cross reactivity it was proposed that the antibody straddles a backbone and interacts with bases in both grooves of the helix.

g) Induction of antibodies against cruciform junctions

Sequences in DNA having inverted repeats give rise to a new structure generally known as "cruciform junction" (Wells et al., 1989; Lilley, 1983). Antibodies specific for cruciforms would be valuable reagents for locating or probing whether such type of structures do form in nature. This type of conformer is not suitable for immunization. Instead, another heteroduplex was constructed by Frappier et al. (1987) that would form a stable cruciform junction. Two monoclonal antibodies (an IgG1 and an IgM) were generated that selectively reacted with the cruciform junction constructed by Frappier et al. (1987).

Objective of Present Study

The present investigation involves the photolinking of a bifunctional photosensitizer 8-methoxypsoralen to calf thymus DNA and poly(dA-dT).poly(dA-dT). Calf thymus DNA obtained commercially was purified free of proteins and single stranded regions. The synthetic copolymer poly(dA-dT) and ctDNA was photoconjugated to 8-methoxypsoralen under UV-A light (320-400 nm). The nature of adduct formed (mono-/diadduct) as a result of photoaddition was investigated by various physico-chemical techniques like ultraviolet and fluorescence spectroscopy nuclease S1, Bal 31 sensitivity, thermal denaturation and hydroxyapatite column chromatography. The separation and identification of modified bases was achieved by DEAE Sephadex A 50 chromatography. The

immunogenicity of photoadducts purified through hydroxyapatite column was ascertained by induction of antibodies in experimental animals. The binding specificity of induced antibodies was characterized by immunodiffusion, direct binding and competition-inhibition ELISA, quantitative precipitin titration and gel retardation assay. The changes induced in nucleic acid polymers as a result of adduct formation was studied by monoclonal anti-ZDNA antibody (Z22).

II EXPERIMENTAL

A. MATERIALS

1. Chemicals

8-Methoxypsoralen, anti-rabbit, anti-goat and anti-human IgG alkaline phosphatase conjugate, poly(dA-dT).poly(dA-dT), xylene cyanole FF, Coomassie Brilliant Blue R 250 and G 250, bovine serum albumin, methylated bovine serum albumin, calf thymus DNA, poly-D-lysine, poly-L-glutamate, dialysis tubings, Freund's complete and incomplete adjuvants, nuclease S1, trinitrobenzene sulfonic acid (TNBS), Tris-(hydroxymethyl)-aminomethane, ethidium bromide, standard protein markers and nuclease Bal 31 were purchased from Sigma Chemical Company, U.S.A. Poly(rG).poly(dC), poly(dG).poly(dC), Sepharose 4B, DEAE Sephacel, Sephadex G 200, agarose NA and Ficoll 400 were obtained from Pharmacia Fine Chemicals, Sweden. Tween-20, acrylamide, bisacrylamide, N-N-N'-N'-tetraethylmethylene-diamine, ammonium persulphate and hydroxyapatite were the products of Bio-Rad Laboratories, U.S.A. Cyanogen bromide and agarose was from S.R.L., India. Acetaldehyde was from Fluka, Switzerland. Perchloric acid and sodium azide was a product of Ferak-Berlin, Germany. Acetonitrile was from E.Merck, India. Polystyrene flat bottom plates having 96 wells (7 mm in diameter) were obtained from Dynatech U.S.A. A colorigenic substrate p-nitrophenyl phosphate was obtained from C.S.I.R. Centre for Biochemicals, Delhi. Psoralen was obtained from C.D.R.I. Lucknow. All other chemicals were of highest grade available commercially.

2. Equipment

Bausch and Lomb Spectronic-20, fraction collector

FRAC-100 (Pharmacia, Sweden), Dynatech ELISA microplate reader MR-600, ELICO pH meter model L1-10T, Shimadzu UV-240 spectrophotometer equipped with a thermoprogrammer and controller, Shimadzu spectrophotofluorometer RF-540, gel electrophoresis apparatus GNA-100 (Pharmacia, Sweden), desk top microfuge RM-12C (REMI, India) and ultraviolet lamp having maximum emission at 365 nm (Vilber Lourmat, France) were the major equipment used in this study.

B. METHODS

1. Purification of Calf Thymus DNA

Highly polymerized calf thymus DNA obtained commercially was purified as described by Ali et al. (1985). DNA (2 mg/ml) dissolved in 0.1x SSC buffer (0.015 M sodium citrate, pH 7.3 containing 0.15 M sodium chloride) was mixed with equal volume of chloroform-isoamyl alcohol (24:1) in a sterile stoppered container. The contents were gently shaken for one hr. The aqueous layer containing DNA was separated from the organic layer and extracted again with chloroform-isoamyl alcohol. The deproteinized DNA was precipitated with two volumes of cold 95% ethanol and collected on glass rod by gentle swirling. The DNA thus obtained was air dried to remove the traces of ethanol and was dissolved in 0.03 M acetate buffer, pH 5.0 containing 0.03 M zinc chloride. The sample was digested by incubating with single strand specific enzyme, nuclease S1 (200 units/mg DNA) at 37^{o}C for 30 min. The reaction was terminated by the addition of one-tenth volume of 0.2 M EDTA, pH 8.0. The purified sample was extracted twice with chloroform-isoamyl alcohol. Finally, the DNA was precipitated with two volumes of cold 95% ethanol. The precipitate was air dried and dissolved in 0.01 M sodium phosphate buffer, pH 7.4 containing 0.15 M sodium chloride. The double strandedness of purified DNA was ascertained by sharp

melting of duplex (Fig. 2)

2. Determination of DNA Concentration

The colorimetric method of Burton (1956) using diphenylamine reagent was employed for the determination of DNA concentration.

a) Preparation of diphenylamine reagent

750 mg of diphenylamine was added to 50 ml of glacial acetic acid followed by the addition of 0.75 ml of concentrated sulphuric acid. The reagent was prepared immediately before use.

b) Procedure

One ml of purified DNA solution was mixed with 1.0 ml of 1 N perchloric acid. The contents were mixed thoroughly and incubated for 15 min in a water bath maintained at 70°C. Hundred uL of 5.43 mM acetaldehyde was added to each assay tube. Two ml of freshly prepared diphenylamine reagent was then added. The contents of each tube were thoroughly mixed and allowed to stand at room temperature for 16-20 hr and the absorbance was recorded at 600 nm. The DNA concentration in unknown sample was determined from the standard plot constructed by using 0-100 ug of purified calf thymus DNA (Fig. 3).

3. Determination of Protein Concentration

Protein was estimated by the method of Lowry et al. (1951) and Bradford (1976).

(i) Estimation of protein by Folin-phenol reagent

The protein estimation by this method involves the complexing of protein with Cu^{2+} in alkaline solution. In addition, the copper appears to catalyze the reduction, by the tyrosine and tryptophan residues of the phospho-molybdate/phosphotungstate anions in the Folin-phenol reagent, added subsequently. This later reaction leads to

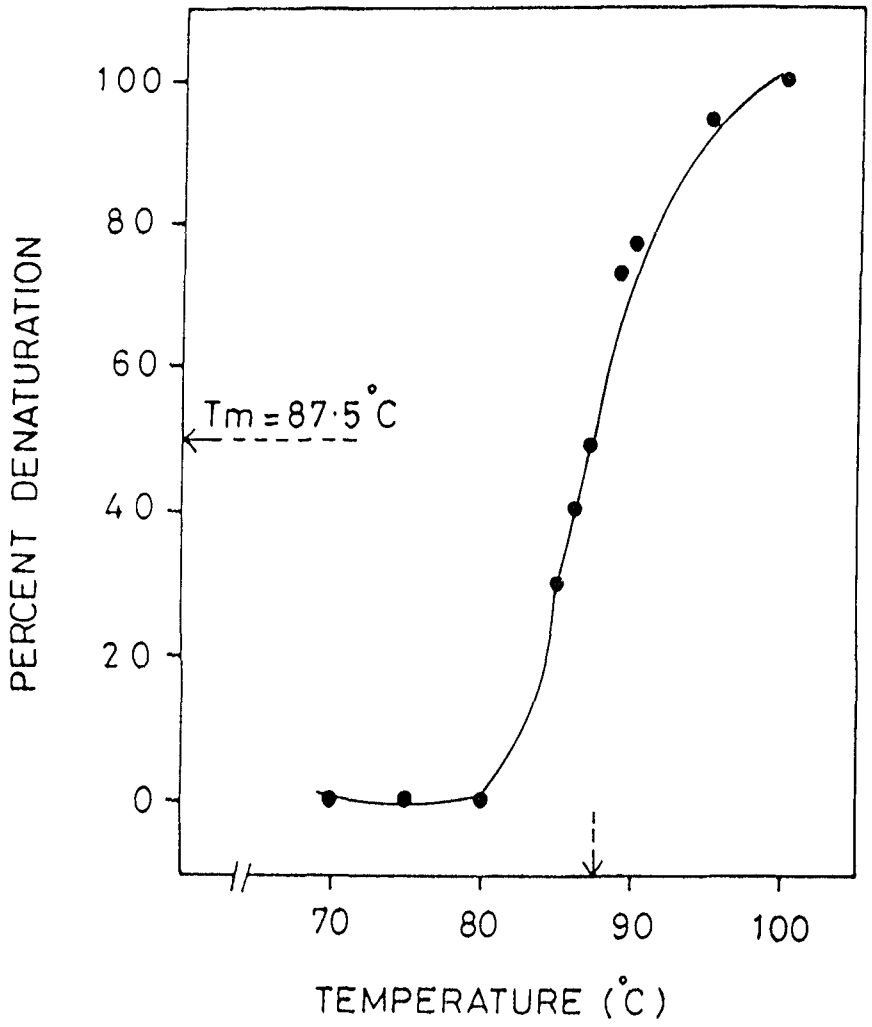


Fig.2. Melting profile of calf thymus DNA in PBS.

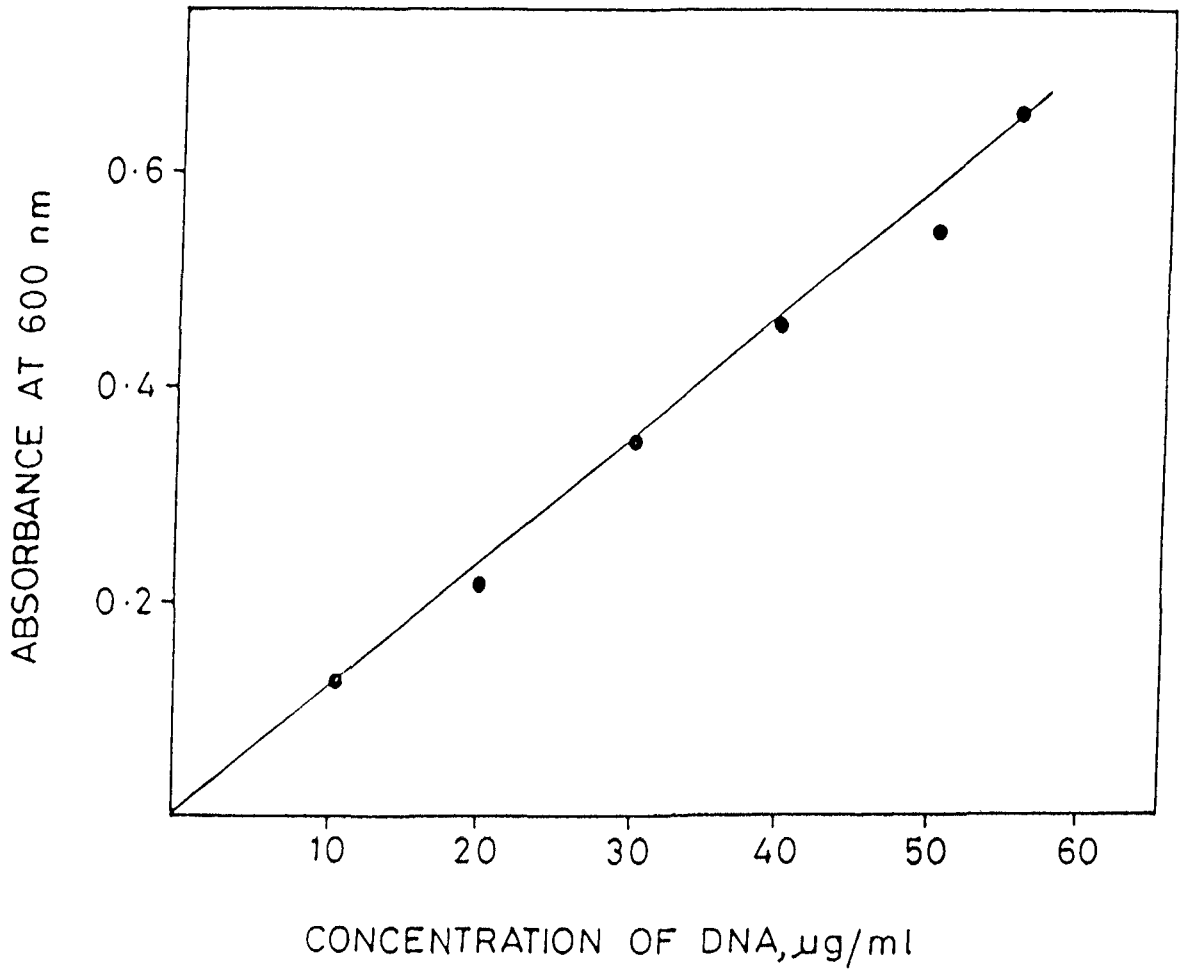


Fig.3.Standard plot for the estimation of DNA by diphenylamine reagent.

a blue colour, which can be measured at 660 nm.

a) Folin-Ciocalteu reagent

The reagent was purchased from C.S.I.R. Centre for Biochemicals, Delhi. The reagent was diluted 1:4 with distilled water before use.

b) Alkaline copper reagent

The components of alkaline copper reagent were:

i) 2% sodium carbonate in 0.1 N NaOH

ii) 1% copper sulphate in 2% sodium potassium tartrate

The working reagent was prepared fresh before use by mixing components (i) and (ii) in the ratio of 50:1.

c) Procedure

One ml of protein sample was mixed with freshly prepared 5.0 ml of alkaline copper reagent. The tubes were allowed to stand at room temperature for 10 min in order to complete the reaction. One ml of 1:4 times diluted Folin-Ciocalteu reagent was added with immediate mixing. After the lapse of 30 min, absorbance was recorded at 660 nm. The concentration of unknown protein sample was computed from standard plot constructed with bovine serum albumin. (Fig. 4).

(ii) Estimation of protein by dye binding

The methodology is based on the change in colour that occurs when Coomassie Brilliant Blue G 250 binds to protein in acidic solution. The protonated form of Coomassie Brilliant Blue dye is pale orange-red in colour. The dye binds strongly to proteins, interacting both hydrophobically and at positively charged (basic) groups on the protein. In the environment of these positively charged groups protonation is suppressed, resulting in the formation of blue colour.

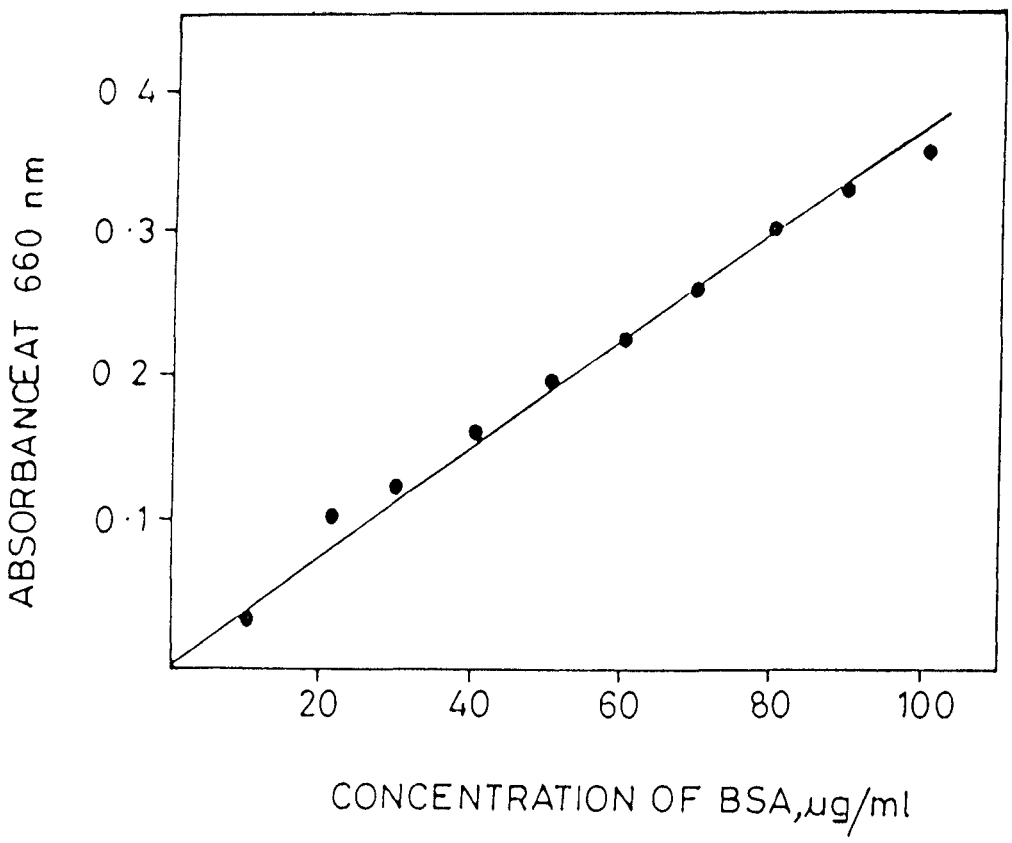


Fig.4. Standard plot for the estimation of protein by Folin-Ciocalteu reagent.

a) Preparation of dye

One hundred mg of Coomassie Brilliant Blue G 250 dye was dissolved in 50 ml of 95% ethanol and filtered to remove the undissolved residues. The dissolved dye was brought to acidic medium by adding 100 ml of 85% (v/v) orthophosphoric acid and was diluted to a final volume of 1 litre with double distilled water.

b) Assay procedure

Appropriate dilutions of protein samples were pipetted in test tubes and the final volume was adjusted to 0.1 ml with buffer. Five ml of dye reagent was added and the contents of each tube were thoroughly vortexed. The reaction was allowed to proceed at room temperature for 15 min and the absorbance was recorded at 595 nm against a reagent blank. The colour is stable for 30 min, after which the precipitation of dye-protein complex may occur. The concentration of protein in an unknown sample was determined from the plot constructed with bovine serum albumin (0-100 ug).

4. Photomodification of Nucleic Acids with 8-Methoxypsoralen

The bifunctional skin photosensitizer, 8-methoxypsoralen was covalently photolinked with calf thymus DNA as well as synthetic polymer, poly(dA-dT).poly(dA-dT) in a controlled two step process.

Calf thymus DNA and poly(dA-dT).poly(dA-dT) in TNE buffer (0.01 M Tris, 0.0004 M EDTA, 0.05 M NaCl) pH 7.6 were mixed separately with 8-MOP in the ratio of 3.2:1 (w/w) in a total volume of 1.0 ml. The mixture was kept in dark for 4 hr at room temperature with constant stirring. The intercalated complexes were irradiated for 40 min using illuminating wavelength of 365 nm. The covalent photoadducts thus formed were extensively dialyzed against 0.01 M Na-Pi buffer (pH 6.8) to remove

unbound drug. Unirradiated samples of DNA, poly(dA-dT).poly(dA-dT) and 8-MOP served as corresponding controls. The occurrence of photoreaction and the formation of photoadduct was studied by ultraviolet and fluorescence spectroscopy.

5. Determination of Melting Temperature

Thermal denaturation of calf thymus DNA, DNA-8MOP photoadduct (in TNE buffer, pH 7.6), poly(dA-dT).poly(dA-dT) and poly(dA-dT)-8MOP photoconjugate (in 0.01 M Na-Pi, pH 6.8) was induced by a temperature scan from 30°C to 95°C at a rate of 1°C/min on Shimadzu UV-240 spectrophotometer equipped with temperature programmer and controller (Hasan and Ali, 1990). Melting curves were recorded at a fixed wavelength of 260 nm. Since Tm is dependent upon the ionic strength of the solvent and the nature of counterions, a single solvent was chosen for test and control samples. The percent denaturation was evaluated by the equation:

$$\text{Percent denaturation} = \frac{A_T - A_{30}}{A_F - A_{30}} \times 100$$

Where,
A_T is the sample absorbance at various temperatures
A_F is the final absorbance at 95°C
A_{30} is the absorbance prior to heating

The change in melting temperature (ΔTm) was calculated using the formula:

$$\Delta Tm = Tm^C - Tm^S$$

where Tm^C and Tm^S are the melting temperatures of native and modified sample respectively.

6. Detection of Photoadduct by Nuclease S1 Sensitivity Assay

Nucleic acid-8MOP photoadducts were also characterized by nuclease S1 treatment (Matsuo and Ross, 1987) followed by agarose gel electrophoresis.

a) Preparation of gel

0.3 gm of agarose in 30 ml of TAE buffer, (0.04 M Tris acetate, pH 8.0 containing 0.002 M EDTA) was brought to molten state by boiling. The solution was allowed to cool to 50-60^{o}C and was poured onto a horizontal tray of GNA-100 electrophoresis apparatus (Pharmacia, Sweden). The poured solution was left at room temperature for 1 hr for complete solidification. The casted gel was 4 mm thick with wells 3 mm deep and 3 mm wide. The capacity of each well was 9 uL and the electrode buffer was TAE.

b) Digestion of photoadduct with nuclease S1

Two ug each of modified and unmodified nucleic acids were incubated with nuclease S1 (20 units/ug of nucleic acid) in acetate buffer, pH 5.0 at 37^{o}C for 30 min. The reaction was stopped by the addition of one-tenth volume of 0.2 M EDTA, pH 8.0.

c) Sample preparation

The loading buffer consist of 0.025% xylene cyanole, 30% Ficoll 400, 0.5 M EDTA in 10 x electrophoresis buffer. The digested and undigested samples contained one-tenth volume of above solution.

d) Running condition

40 volts for 2 hr.

e) Staining

The DNA bands were visualized by staining with

ethidium bromide (1 ug/ml).

7. Hydroxyapatite Column Chromatography

Hydroxyapatite possesses a unique property of distinguishing between single stranded and double stranded conformations of nucleic acids and thus it was employed to ascertain the mono- or diadduct nature of photomodified nucleic acid polymers.

The commercial sample of hydroxyapatite was washed several times with 0.01 M sodium phosphate (Na-Pi) buffer, pH 6.8 to remove the fine particles and thereafter packed in a glass column (1.5 cm x 6.0 cm). After equilibration with 0.01 M Na-Pi buffer, pH 6.8, heat denatured photoadducts (DNA-8MOP and poly(dA-dT)-8MOP) in equilibrating buffer were adsorbed onto the column. Control samples of native and heat denatured nucleic acid polymers (DNA and poly(dA-dT)) were also subjected to hydroxyapatite column chromatography under identical conditions. Stepwise elution was carried out with 0.125 M and 0.25 M Na-K- phosphate buffer, pH 6.8 (Dardalhon and Averbeck, 1988). Fractions of 3.0 ml were collected at a flow rate of 14 ml/hr. Single stranded DNA was eluted with 0.125 M Na-K-phosphate buffer, pH 6.8. Heat denatured photoadducts and double stranded DNA were found to be eluted with 0.25 M Na-K-phosphate buffer, pH 6.8. All samples were denatured (80 ug/ml) by heating in a boiling water bath for 15 min and fast cooled in ice-NaCl bath. The fractions eluted as diadduct were pooled and used as antigen.

8. Bal 31 Digestion of Photoadducts

The study was based on the digestion of double stranded DNA by the exonuclease Bal 31, which was found to be arrested at psoralen-DNA interstrand crosslinked sites (Zhen et al., 1986). DNA samples were

electrophoresed by the procedure of Sealey and Southern (1985). The following stock solutions were prepared:

i) 40% acrylamide in distilled water
ii) 2% bisacrylamide in distilled water
iii) Gel buffer: 0.9 M Tris borate, pH 8.3 containing 0.025 M EDTA.
iv) Electrode buffer: 0.09 M Tris borate, pH 8.3 containing 0.0025 M EDTA

Recipe for 6% resolving gel
(Total volume 40.0 ml)

40% acrylamide	6.0 ml
2% bisacrylamide	6.0 ml
Gel buffer	4.0 ml
1.5% ammonium persulphate	2.0 ml
Distilled water	22.0 ml
TEMED	0.025 ml

Photoadduct, hydroxyapatite purified photoadduct and calf thymus DNA (1.0 ug each) in Bal 31 buffer (0.6 M NaCl, 0.02 M Tris, 0.012 M $MgCl_2$, 0.012 M $CaCl_2$, 0.001 M EDTA) pH 8.0 were treated with 0.5 units of Bal 31 for 0, 5, 10 and 20 min at 30^{o}C. The reaction was stopped by the addition of one-tenth volume of 0.2 M EDTA, pH 8.0. The DNA samples were run for 6 hr at 70 volts after the addition of 3.0 uL of "stop mix" (30% Ficoll 400, 0.025% xylene cyanole FF, 0.5 M EDTA in 10 x electrophoresis buffer). On completion of electrophoresis, the gel was stained with ethidium bromide (1 ug/ml) and DNA bands were visualized under UV illumination.

9. Separation and Quantitation of Photomodified Bases

The separation, quantitation and identification of the drug (8-MOP) photoconjugated to bases was

accomplished as described earlier (Hasan and Ali, 1990).

a) Acid hydrolysis of DNA-8MOP photoadduct

The photoadduct was precipitated with two volumes of cold ethanol and was collected after centrifugation at 5000 rpm for 10 min. The precipitate was dessicated in order to remove the traces of ethanol and dissolved in perchloric acid (60%). The sample was treated in a boiling water bath for 1 hr to release the bases. The solution was neutralized and concentrated.

b) DEAE Sephadex A 50 column chromatography

The separation of bases was performed by ion exchange chromatography on DEAE Sephadex A 50 column. The supplied gel was swelled by keeping at 90^{o}C for 1 hr in distilled water. The gel was packed in a column (1.6 cm x 30 cm) and left overnight for gravity packing. The column was equilibrated with copious volume of 0.02 M Tris buffer, pH 7.2. Sample (2.5 ml) was applied onto the column with the aid of an applicator and eluted with same buffer at a flow rate of 40 ml/hr. Two hundred fractions of 2.5 ml each were collected and their absorbance recorded at 260 nm. The control experiment was carried out with hydrolyzed calf thymus DNA. The individual bases were identified by their characteristic UV absorption profile.

10. Preparation of Antigen and Immunization Schedule

a) Antibodies against poly(dA-dT)-8MOP photoadduct

Poly(dA-dT)-8MOP-MBSA conjugate was formed by mixing equal amounts of both poly(dA-dT)-8MOP and MBSA by weight of the solutes. One ml of poly(dA-dT)-8MOP photoadduct (100 ug) was complexed with 100 uL of MBSA (100 ug). The complex was emulsified with equal volume of Freund's complete adjuvant for the first injection. All subsequent

injections were in incomplete adjuvant. Goat was injected intramuscularly in hind limbs weekly for five weeks (Ishaq and Ali, 1984). A single animal received a total of 500 ug of photoadduct in the course of five injections. Ten days after the last dose, animal was bled through jugular vein. The serum samples were heated at 56°C for 30 min to inactivate complement which inhibits the formation of and also dissolves preformed antigen-antibody complexes (Miller and Nussenzweig, 1975; Schifferli et al., 1980). The decomplemented samples were stored at -20°C with 0.02% sodium azide as preservative. Animal was also bled prior to immunization to obtain preimmune serum.

b) Antibodies against DNA-8MOP photoadduct

Rabbits (8-12 months old, weighing 1.0 to 1.5 kg) were injected intramuscularly in hind limbs with freshly prepared solution of DNA-8MOP photoadduct (50 ug) and MBSA (50 ug) in Freund's complete adjuvant in a total volume of 1.0 ml. Blood was collected by cardiac puncture prior to immunization (Ali and Ali, 1983). Subsequent injections were given weekly for four weeks with the same amount of antigen but in Freund's incomplete adjuvant. Each animal received a total of 250 ug of antigen in the course of five injections. A week after the last injection blood was drawn by cardiac puncture and serum separated. The Clq complement component was heat inactivated by keeping serum samples at 56°C for 30 min. Serum was aliquoted in small volumes and kept at -20°C with 0.02% sodium azide as preservative.

c) Sera

Normal sera were obtained from healthy subjects. SLE sera were obtained from patients showing high titer anti-DNA antibodies and fulfilled the American Rheumatism Association Criteria for this disease (Tan et al., 1982). SLE negative and positive controls were obtained from

Sigma, U.S.A. All sera were heat inactivated (56°C, 30 min) and stored at -20°C with 0.02% sodium azide as preservative. Specific monoclonal anti-ZDNA antibody Z22 (IgG) was a generous gift from Dr. B.D. Stollar (U.S.A.).

11. Isolation of IgG

The preimmune as well as immune IgG were isolated from their respective serum by DEAE Sephacel column chromatography of 35% saturated ammonium sulphate precipitated immunoglobulin fractions (Ishaq and Ali, 1987).

a) Preparation of crude IgG

Saturated ammonium sulphate was added dropwise to 6.5 ml serum, allowing each drop to disperse before the next was added. Most of the immunoglobulins got precipitated by 35-40% of saturation. The mixture was allowed to stand at 4°C for 1 hr in order to attain complete precipitation. The suspension was stirred for 15-30 min and then centrifuged at 10,000 rpm for 15 min. The pellet thus obtained was washed three times with 40% saturated ammonium sulphate. The washed precipitate was dissolved and dialyzed against 500-1000 volumes of 0.01 M sodium phosphate buffer, pH 8.0. The dialysis fluid was changed several times at intervals of few hr.

b) DEAE Sephacel chromatography

The crude immunoglobulins were loaded onto a DEAE Sephacel column (1.5 cm x 28 cm) preequilibrated with 0.01 M sodium phosphate buffer, pH 8.0. The column was washed with 0.01 M sodium phosphate buffer, pH 8.0 until the absorbance at 280 nm reached base line. Fractions of 3.0 ml were collected with a linear gradient of 0.01 M - 0.3 M sodium phosphate buffer, pH 8.0. Absorbance of each fraction was monitored at 280 nm. First peak of the chromatogram was pooled and used as IgG.

12. Purification of Isolated IgG

Pure preparations of immune IgG was obtained by performing a preparative gel chromatography on Sephadex G 200 column (2 cm x 75 cm). The column was packed according to the instructions provided by the manufacturer. Fifty mg of DEAE Sephacel isolated IgG in a total volume of 3.0 ml was loaded onto the pre-equilibrated column. Fractions of 4.0 ml were collected with 0.01 M sodium phosphate buffer, pH 7.4 containing 0.15 M sodium chloride at a flow rate of 20 ml/hr. The protein content of each fraction was monitored at 280 nm. The fractions showing A_{278}/A_{251} ratio of 2.5 and single band in SDS-PAGE were pooled, dialyzed against appropriate buffer and used as purified IgG. The pooled IgG was stored at -20°C in small aliquots with 0.02% sodium azide as preservative.

13. SDS-Polyacrylamide Gel Electrophoresis

The homogeneity of purified IgG was ascertained by polyacrylamide slab gel electrophoresis as described by Laemmli (1970) under denaturing conditions.

a) Acrylamide-bisacrylamide (30:0.8)

The stock solution was prepared by dissolving 30 gm of acrylamide and 0.8 gm of bisacrylamide in a total volume of 100 ml. The solution was stored at 4°C in light protective bottles.

b) Resolving gel buffer

Stock buffer solution was prepared by dissolving 36.3 gm Tris in 48.0 ml of 1 N HCl (3.0 M). The contents were mixed properly, pH adjusted to 8.8 and final volume brought to 100 ml with distilled water.

c) Stacking gel buffer

6.05 gm Tris was dissolved in 40.0 ml distilled

water, titrated to pH 6.8 with 1 N HCl (around 48 ml) and the volume adjusted to 100 ml with distilled water (0.5 M).

d) Electrode buffer

0.025 M Tris and 0.192 M glycine, pH 8.3 containing 0.1 percent SDS.

e) Sample buffer

i) 6.0 gm Tris was dissolved in 80 ml distilled water and pH adjusted to 6.8 with phosphoric acid. The volume was made to 100 ml with distilled water.

ii) To 12.5 ml of the above sample buffer was added 1 mg bromophenol blue and 12.5 ml glycerol.

One part of (ii) and four part of the sample were mixed prior to electrophoresis and heated in a boiling water bath for 3 min. This ensures denaturation of protein. Samples were electrophoresed at 80 V at room temperature for 8-10 hr.

Recipe for 7.5% SDS-PAGE
(Total volume 30.0 ml)

Acrylamide-bisacrylamide	7.5	ml
Resolving gel buffer	3.75	ml
10% SDS	0.30	ml
1.5% ammonium persulfate	1.5	ml
Distilled water	16.95	ml
TEMED	0.015	ml

Recipe for stacking gel buffer
(Total volume 10 ml)

Acrylamide-bisacrylamide	1.25	ml
Stacking gel buffer	2.50	ml
10% SDS	0.10	ml

1.5% ammonium persulfate	0.50 ml
Distilled water	5.65 ml
TEMED	0.075 ml

The resolving gel buffer was mixed and poured between the glass plates separated by 1.5 mm thick spacer. The gel was allowed to polymerize at room temperature. The stacking gel was added thereafter. The samples were loaded, electrophoresed and stained with Coomassie Brilliant Blue R 250 in 25% isopropyl alcohol and 10% glacial acetic acid. Destaining was carried out in a mixture of 10% acetic acid and 10% methanol.

14. Immunoaffinity Purification of Antibodies

Epitope specific paratopes in the heterogeneous immune sera were obtained by affinity purification of induced antibodies on polylysyl-Sepharose 4B coupled with the immunogen (Hasan et al., 1991).

a) Activation of matrix

Fifteen ml of Sepharose 4B slurry obtained from Pharmacia was suspended in distilled water, filtered and washed with 300 ml of double distilled water on a sintered glass funnel (porosity G 2). Ten gm of moist gel, mixed with 10 ml of 2 M sodium carbonate was kept in an ice-NaCl bath placed on a magnetic stirrer. A total of 0.8 ml acetonitrile having 1.0 gm CNBr was added dropwise to the gel and contents were allowed to interact for 12 min in cold. The reaction mixture was filtered through sintered glass funnel and washed with 400 ml of cold 0.1 M sodium bicarbonate (coupling buffer) and resuspended in same buffer. The unreacted CNBr (drained effluent) was reacted with ferrous sulphate to convert it into harmless ferrocyanide. All steps were carried out in fume-hood chamber (Ali, 1984).

b) Coupling of poly-L-lysine with activated Sepharose 4B

The method described by Wilchek (1973) was followed. The polycationic homopolypeptide polylysine (100 mg) was brought into solution form by dissolving in 10.0 ml of 0.1 M sodium bicarbonate and was added to the gel immediately after CNBr activation. The mixture was kept at $4^{o}C$ for 12 hr with constant but slow stirring. The buffer was drained out and the gel was washed successively with 100 ml each of cold (i) distilled water (ii) 0.1 N HCl (iii) 0.1 M sodium bicarbonate and (iv) distilled water till neutral. Finally the gel was resuspended in 40 ml of 0.15 M acetate buffer, pH 4.5. The extent of poly-L-lysine depletion due to covalent coupling with activated Sepharose 4B was quantified by treatment of drained effluent with TNBS as described by Habeeb (1966).

c) Affinity purification of anti-photoadduct antibodies

DNA-8MOP-[polylysyl-Sepharose 4B] column was prepared as described by Nicotra et al. (1982) with slight modification. Twenty ml of polylysyl-Sepharose 4B was packed in a glass minicolumn (1.6 cm x 5.0 cm) and equilibrated with acetate buffer. DNA-8MOP photoadduct (12.5 ml of 100 ug/ml in acetate buffer) was applied onto the preequilibrated column and subjected to recycling for a couple of times in order to ensure maximum electrostatic binding of the photoadduct to the matrix. The unbound material was washed with 50 ml of PBS, pH 7.4. IgG isolated from anti-photoadduct serum, dialyzed previously against PBS, was loaded onto the affinity column. The unbound antibodies were removed by washing the column with 40 ml of PBS. The bound antibodies were eluted with 0.3 M NaCl in PBS, pH 7.4. Fractions of 3.0 ml were collected at a flow rate of 18 ml/hr. The contents of each tube were monitored at 251 nm, 260 nm, 278 nm and 280 nm.

d) Regeneration of affinity column

For further use, the column was regenerated by washing successively with 50 ml each of the following:

i) Cold distilled water

ii) Cold 0.1 N HCl

iii) Cold 0.1 M $NaHCO_3$ and

iv) Cold distilled water till neutral

15. Immunological Techniques

a) Immunodiffusion

The method described by Tan et al. (1966) was followed. The solid phase was 0.4% agarose in PBS, pH 7.4.

i) Preparation of agarose petri dishes

Six ml of 0.4% molten agarose in PBS containing 0.1% sodium azide was poured into a 5 cm x 1.5 cm glass petri dishes and allowed to solidify at room temperature. Wells, each 5 mm in diameter, were made with the help of a gel puncture and were separated from each other by a distance of 8 mm. The agarose layered petri dishes were stored at $4^{o}C$.

ii) Assay procedure

Twenty five uL of serially diluted decomplemented serum and antigen were placed in wells. The petri dishes were allowed to stand in a moist chamber at room temperature for 48-72 hr. The gels were washed with 5% sodium citrate to eliminate non specific precipitin lines. The precipitin lines were analyzed visually and the results recorded.

b) Quantitative precipitin titration

An attempt was made to investigate the immunointeraction of the induced antibodies with polyvalent immunogen having distinct conformation by

means of quantitative precipitin titration. Antigen-antibody interaction was carried out in sterilized Eppendorf microfuge tubes.

Photoadducts of varying concentration (0-50 ug/ 0-25 ug) were allowed to interact electrostatically in Eppendorf tube with a constant amount of immune IgG in an assay volume of 0.2 ml. The interaction was allowed to attain its minimum Van der Waal's radii by incubating the mixture at 37^{o}C for 2 hr followed by overnight incubation at 4^{o}C. The assay tubes were spun in microfuge at 8,000 rpm for 2.5 min. The supernatant was carefully drained out and precipitated immune complex was washed thrice with cold PBS, pH 7.4. The precipitate was dissolved in PBS, pH 7.4 containing 1 M NaCl. The photoadduct and antibody in the dissociated immune complex was estimated by diphenylamine and dye binding assay respectively. The binding data were analyzed and antibody affinity was calculated by Scatchard (1949) as well as Langmuir (1918) isotherm plot.

c) Enzyme linked immunosorbent assay

Preparation of buffers and substrate

i) Tris buffer saline (TBS): 0.01 M Tris, 0.15 M NaCl, pH 7.4.

ii) Tris buffer saline - Tween-20 (TBS-T): 0.02 M Tris, 0.144 M NaCl, 0.00268 M KCl, 500 uL Tween-20, pH 7.4.

iii) Bicarbonate buffer: 0.015 M sodium carbonate, 0.035 M sodium bicarbonate and 0.002 M magnesium chloride, pH 9.6 containing 0.02% sodium azide as preservative

iv) Substrate: 500 ug p-nitrophenyl phosphate/ml in bicarbonate buffer.

Assay procedure

Polystyrene flat bottom microtiter plates were precoated with 100 uL of poly-D-lysine (50 ug/ml in distilled water) for 30 min at room temperature to increase antigen immobilization. The poly-D-lysine coated wells were washed thrice with TBS, pH 7.4. One hundred uL nucleic acid antigens (2.5 ug/ml in TBS) were coated for 2 hr at room temperature followed by overnight incubation at $4^{o}C$. The plates were washed thrice with TBS-T and coated with 100 uL/well of poly-L-glutamate (50 ug/ml in TBS) for 2 hr at room temperature to neutralize the positive charges of unreacted poly-D-lysine. The plates were again washed and finally the unoccupied sites, both in control and antigen coated wells, were saturated with 100 uL of 1% bovine serum albumin (in TBS) for 5 hr at room temperature. The plates were washed once with TBS-T and antibodies (diluted in 1% BSA-TBS) were added both in antigen and control wells. The plates were incubated for 2 hr at room temperature and overnight at $4^{o}C$. The unbound material on the plates was eliminated by washing thrice with TBS-T, pH 7.4 and appropriate anti-IgG-alkaline phosphatase conjugate (1:1500 dilution in TBS) was coated for 2 hr at room temperature. A colorigenic substrate, p-nitrophenyl phosphate was added and the absorbance of each well was recorded at 410 nm on an automatic microplate reader. The results were expressed as a mean of $A_{Test} - A_{Control}$.

d) Competition-inhibition ELISA

The specificity of antigen-antibody interaction was checked by inhibition ELISA (Ali and Ali, 1986). Varying amounts of soluble competitors were incubated with constant amounts of antibody (serum or IgG). The preparation was incubated for 2 hr at $37^{o}C$ and overnight at $4^{o}C$ before they were coated onto the test plates. Rest of the steps were same as in simple ELISA. The inhibition was expressed according to the formula:

$$\text{Inhibition (\%)} = 1 - \left[\frac{\text{(OD inhibited)-(Background)}}{\text{(OD uninhibited)-(Background)}}\right] \times 100$$

16. Gel Retardation Assay

Varying amounts (0-28 ug) of antibodies were incubated with photoadducts (DNA-8MOP or poly(dA-dT)-8MOP) in a total volume of 15 uL for 2 hr at 37°C (Alam and Ali, 1992). The antigen-antibody interaction was stabilized by incubating the mixture overnight at 4°C. After completion of incubation period, 1.0 uL of "stopmix" was added in each assay tube. The immune complex thus formed was electrophoresed on 1% agarose using 0.04 M Tris acetate and 0.002 M EDTA pH 8.0. Control experiments were carried out in presence or absence of preimmune goat IgG. On completion of electrophoresis, the gel was stained with ethidium bromide (1 ug/ml) and photographed under UV illumination.

III RESULTS

Photoaddition of 8-Methoxypsoralen to Native DNA and Poly(dA-dT).poly(dA-dT)

Nucleic acid polymers (native DNA and poly(dA-dT).poly(dA-dT)) in TNE buffer, pH 7.6 were mixed with 8-methoxypsoralen (8-MOP) in the ratio of 3.2:1 (w/w). The mixture was irradiated for 40 min under UV-A light (320-400 nm). The irradiated samples were dialyzed against TNE to remove free drug molecules. The ultraviolet and fluorescence spectra of native and modified nucleic acid polymers were recorded to detect photoadduct formation. The ultraviolet absorption spectra of 8-methoxypsoralen showed three distinct peaks centered at 215, 245 and 300 nm with a shoulder at 261 nm (Fig. 5). Native DNA showed the usual peak at 260 nm with a minimum absorbance at 232 nm. DNA modified with 8-MOP in presence of UV-A light showed hypochromicity at 260 nm and hyperchromicity around 310 nm (Fig. 5). The spectral changes revealed the photoaddition of 8-MOP to DNA.

Figure 6 shows the characteristic UV absorption pattern of 8-MOP and poly(dA-dT).poly(dA-dT). The modification of poly(dA-dT).poly(dA-dT) with 8-MOP under UV-A light showed significant decrease in absorption at 260 nm and increase in absorbance around 310 nm (Fig. 6). However, the increased absorbance around 310 nm is much pronounced in poly(dA-dT) as compared to native DNA modified under identical conditions. These results thereby indicate the formation of photoadducts and that the synthetic polymer poly(dA-dT).poly(dA-dT) is a better substrate for 8-MOP photoaddition.

Native and modified nucleic acid polymers were also analyzed by fluorescence emission spectra. The free 8-MOP showed fluorescence emission maxima at 507 nm when

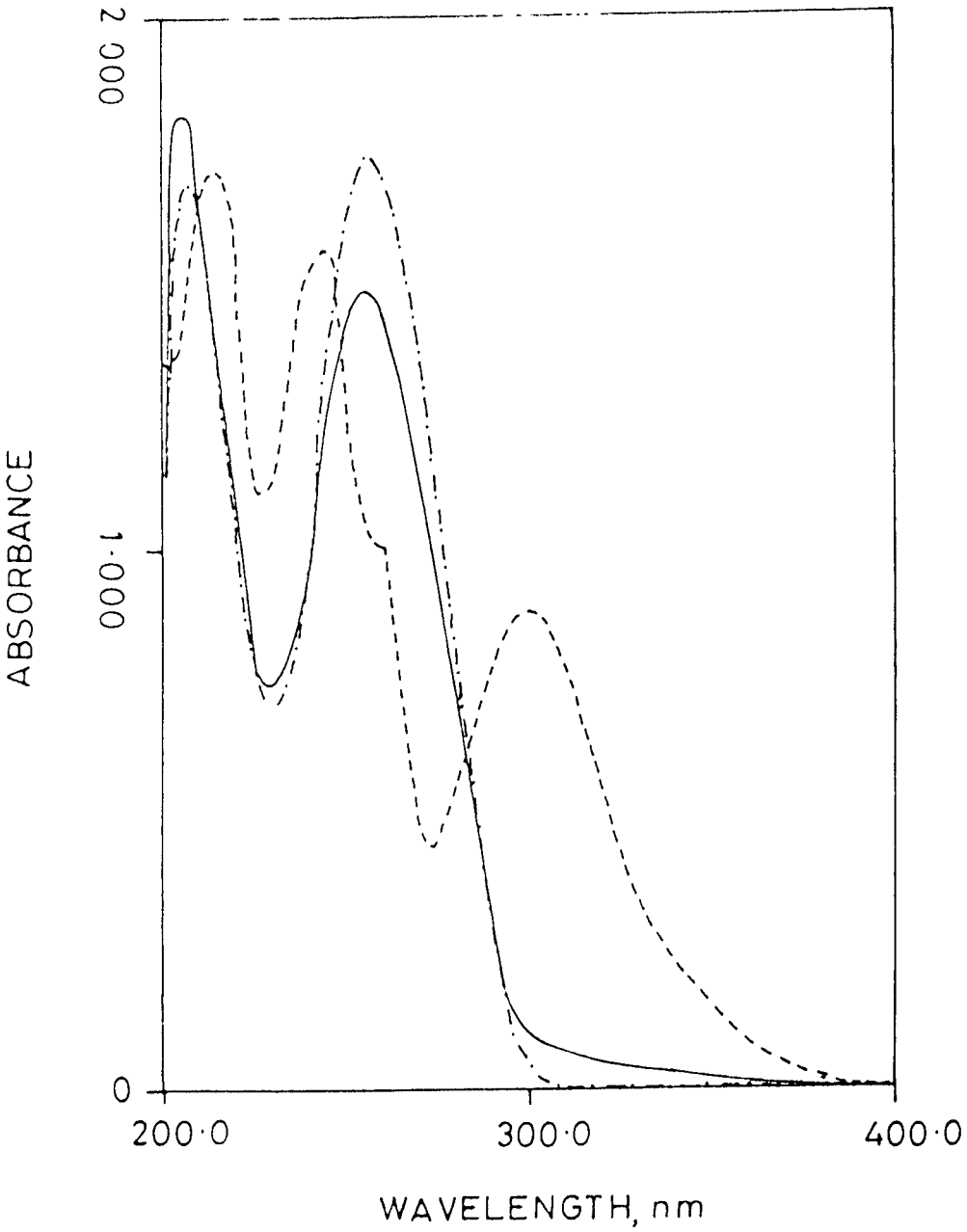


Fig.5. Ultraviolet absorption characteristics of 8-methoxypsoralen (- - - - -), native DNA (—·—·—·) and DNA-8MOP photoadduct (———).

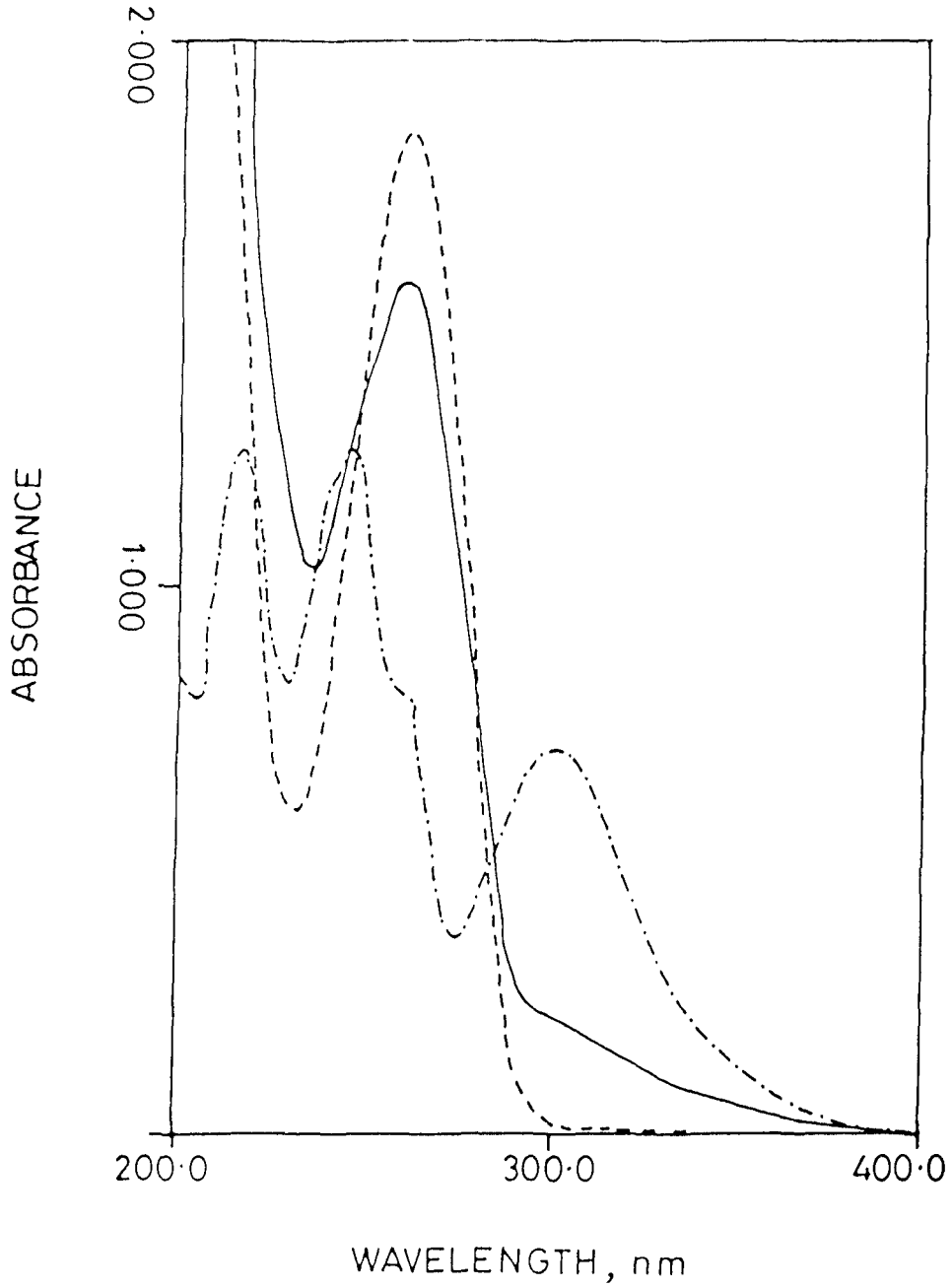


Fig.6. Ultraviolet absorption spectra of 8-MOP (—·—·—·—), poly(dA-dT).poly(dA-dT) (- - - - -) and poly(dA-dT)-8MOP photoadduct (———).

excited at 307 nm. The fluorescence intensity of free 8-MOP dropped appreciably once it got photoreacted with either DNA or poly(dA-dT) (Figs. 7 & 8). Besides significant drop in fluorescence, the emission wavelength of free 8-MOP showed a blue shift from 507 nm to 415 nm upon UV-A irradiation of DNA and poly(dA-dT) in presence of 8-MOP. However, native DNA and poly(dA-dT) did not show appreciable fluorescence in absence of 8-MOP and UV-A light. Table 5 depicts the fluorescence characteristics of native and modified polymers and of 8-MOP. The substantial decrease in fluorescence intensity and the observed blue shift of 92 nm in the photomodified conformers reflect the occurrence of photoreaction between nucleic acid polymers and 8-MOP and consequent formation of the photoadducts.

Temperature Dependent Melting of Photoadducts

Photomodification of nucleic acid polymers as a result of 8-MOP addition under UV-A light was probed by monitoring thermal induced unwinding of double helix. The polymer melting was scanned in the temperature range of 30-95^{o}C after 10 min equilibration at 30^{o}C. The duplexes were melted at the rate of 1^{o}C/min. Increase in ultraviolet absorption at 260 nm was taken as a measure of helix unwinding. As illustrated in Fig. 9, the spectroscopic melting curves for native DNA and DNA-8MOP photoadduct displayed a progressive increase in absorbance from 75^{o}C onward. The mid-point of transition (Tm) was at 78.5^{o}C and 83.5^{o}C for DNA and DNA-8MOP photoadduct respectively. The net increase of 5^{o}C in the melting temperature of photoadduct shows stabilization of double helix by 6.3% as a consequence of adduct formation. The percent hyperchromicity of DNA and DNA-8MOP was 37.4 and 32.5 respectively (Table 6). The observed decrease in percent hyperchromicity at 95^{o}C and increase in Tm of modified DNA indicates the formation of diadduct on photomodification of native DNA.

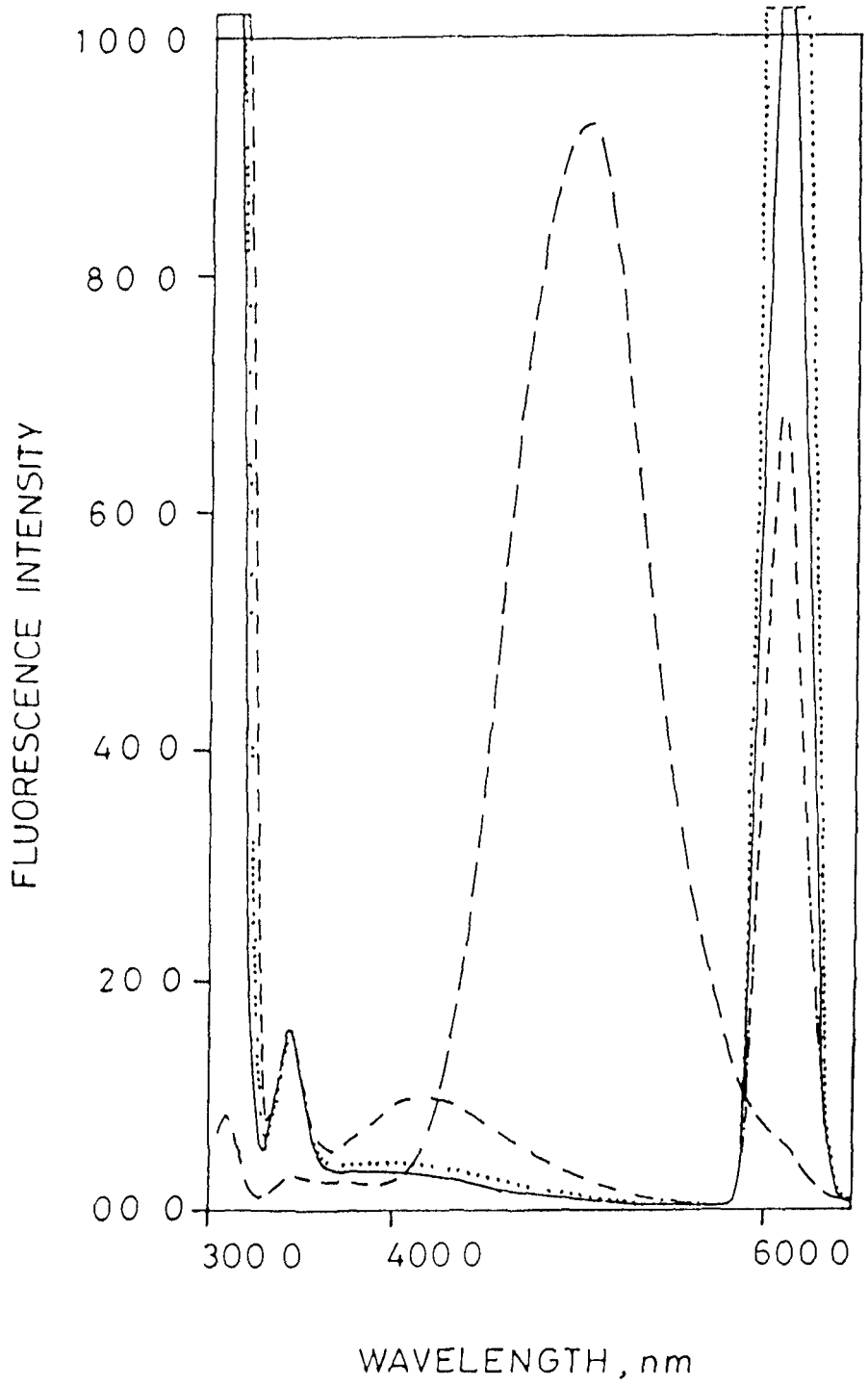


Fig.7. Fluorescence emission characteristics of 8-MOP (— — —), native DNA (·····), DNA-8MOP photoadduct (-·-·-·-) and 0.01 M Na-Pi, pH 6.8 (——).

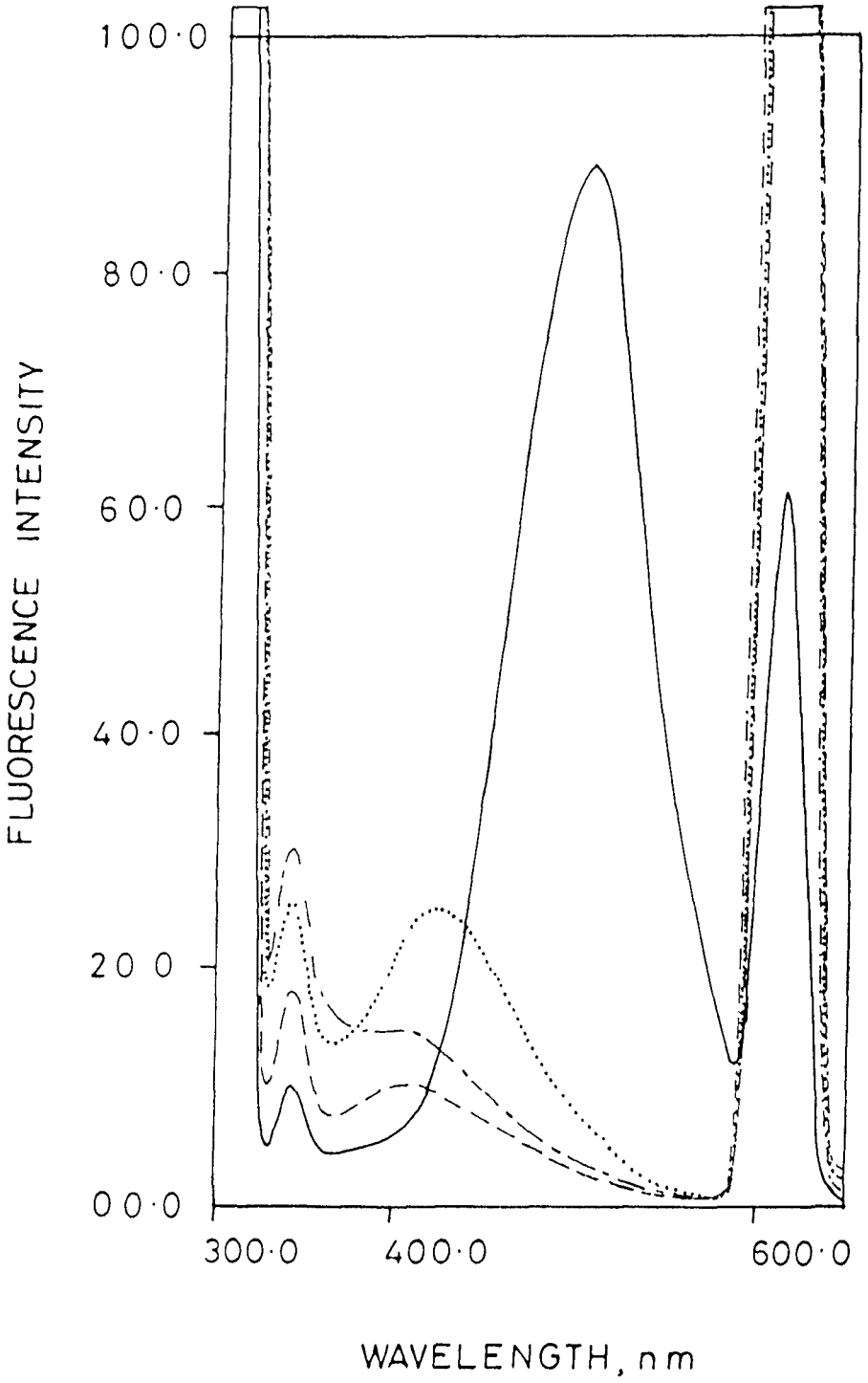


Fig.8. Fluorescence emission spectra of 8-MOP (————), poly(dA-dT) (– – – – –), poly(dA-dT)-8MOP photoadduct (·····) and 0.01 M Na-Pi, pH 6.8 (– – – –).

TABLE 5

Fluorescence emission characteristics of free and bound 8-methoxypsoralen

Drug in free or bound state	Emission wavelength (nm)
8-Methoxypsoralen	507
Native DNA	NIL
DNA-8-MOP	415
Poly(dA-dT)	NIL
Poly(dA-dT)-8MOP	415

Excitation wavelength was 307 nm

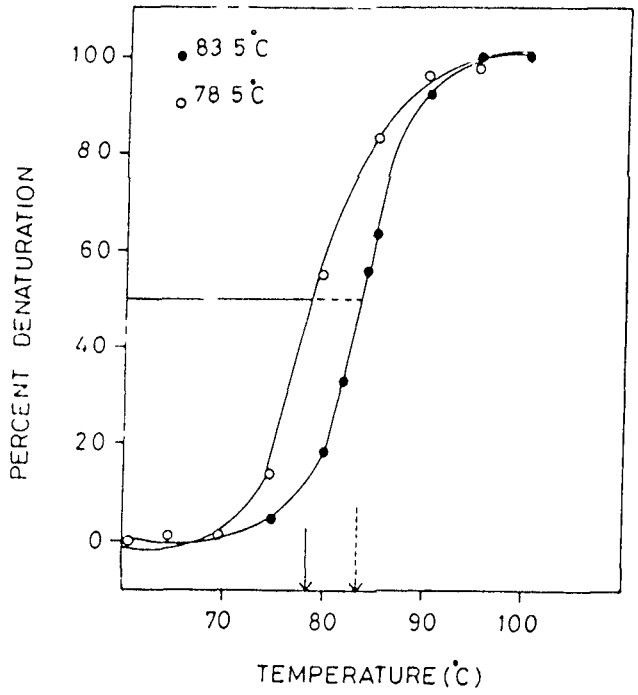


Fig.9. Temperature induced melting of native DNA (—○—) and DNA-8MOP photoadduct (—●—).

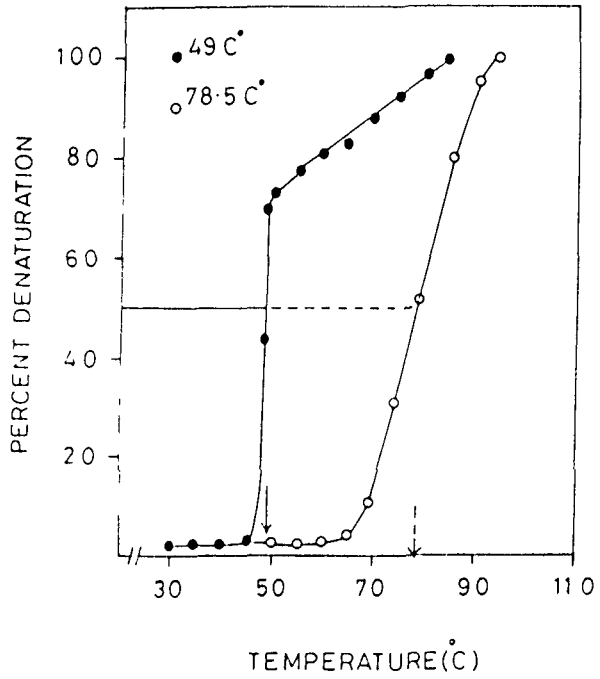


Fig.10. Thermal denaturation profile of native poly(dA-dT). poly(dA-dT) (—●—) and poly(dA-dT)-8MOP photoadduct (—○—).

TABLE 6

Thermal denaturation data of native and photomodified DNA duplex

Polymer	Percent duplex melted at 75°C	Mid-point melting temperature (Tm)	Percent hyperchromicity at 95°C
Native DNA	12.7	78.5	37.4
DNA-8MOP	5.2	83.5	32.5
Poly(dA-dT)	91.4	49.0	56.7
Poly(dA-dT)-8MOP	31.3	78.5	18.6

The covalent linkage of synthetic copolymer poly(dA-dT).poly(dA-dT) with 8-MOP resulted in the Tm value of 78.5°C (Fig. 10). The native polymer melted under identical conditions revealed a Tm value of 49.0°C. The diadduct presence in modified poly(dA-dT)-8MOP complex was evident by 29.5°C increase in melting temperature. The decreased percent hyperchromicity of modified poly(dA-dT) is another evidence of crosslink formation (Table 6).

Nuclease S1 Sensitivity of Photoadducts

DNA nucleases are most widely used tools of modern day molecular biology. Enzymes, such as nuclease S1 and Bal 31 have been used in probing the mono- or diadduct nature of photoadducts (Carlson et al., 1982; Zhen et al., 1986).

The agarose gel electrophoresis of modified DNA polymers under native and heat denatured conditions, in presence or absence of nuclease S1 have been presented in Fig. 11. Native and heat denatured photoadducts showed identical electrophoretic migration patterns. Incubation of one microgram each of native and heat denatured photoadducts with 20 units of nuclease S1 for 30 min at 37°C showed almost equal level of digestion as revealed by its movement in gel. The result could be interpreted as the presence of unstacked base pair regions adjacent to the crosslinking site which are resistant to thermal melting and thus providing a strong evidence for the presence of interstrand crosslinks in the photomodified conformers. By contrast, the heat denatured native polymers were completely digested by the enzyme.

Hydroxyapatite Chromatography of DNA-8MOP and Poly(dA-dT)-8MOP Photoadducts

The separation of monoadducts and crosslinked species of photoadducts was achieved by spontaneous

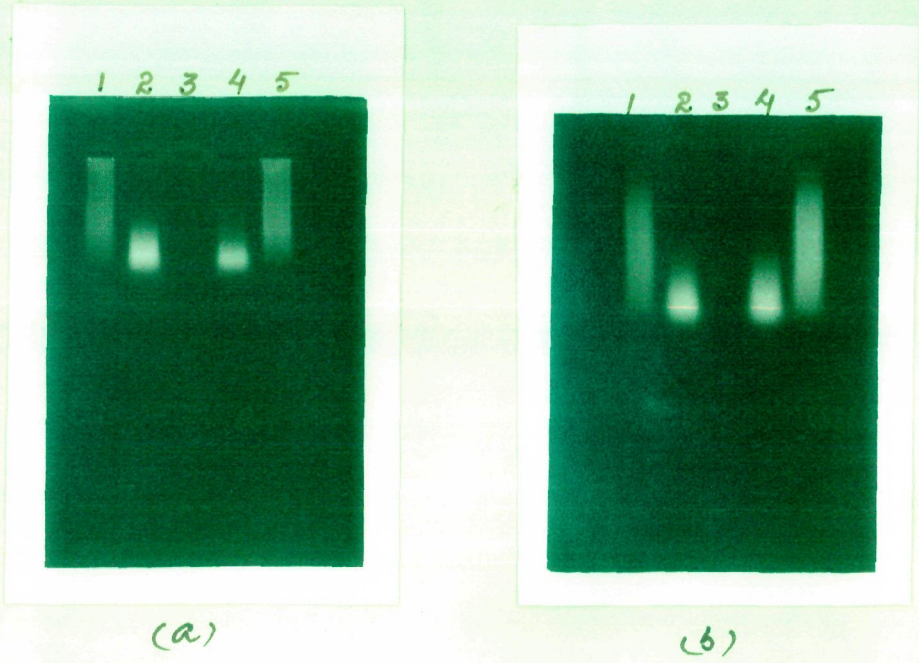


Fig.11.(a). Nuclease S1 sensitivity of poly(dA-dT)-8MOP photoadduct in 1% agarose gel. Poly (dA-dT)-8MOP photoadduct (lane 1), nuclease S1 incubated with: photoadduct (lane 2), denatured polymer (lane 3) and denatured photoadduct (lane 4). Lane 5 shows the electrophoretic mobility of denatured photoadduct.

(b). Nuclease S1 sensitivity of DNA-8MOP photoadduct. After digestion the mixture was subjected to electrophoresis in 1% agarose gel. Heat denatured DNA-8MOP photoadduct (lane 1), nuclease S1 digested: (i) denatured photoadduct (lane 2), (ii) denatured DNA (lane 3) and (iii) photoadduct (lane 4). Lane 5 shows the electrophoretic mobility of photoadduct.

renaturation capacity of interstrand crosslinked DNA polymer after heat denaturation. Native DNA, heat denatured DNA and denatured DNA-8MOP photoadduct were adsorbed on hydroxyapatite column and washed with 0.01 M Na-Pi buffer, pH 6.8 Stepwise elution with 0.125 M and 0.25 M Na-K-phosphate buffer was carried out. The native DNA was eluted with 0.25 M buffer, whereas the desorption of denatured DNA occurred mainly at 0.125 M with a minor population eluting at the molarity of native DNA (Fig. 12). By contrast, denatured photoadduct (DNA-8MOP) showed a major peak at the position of double stranded DNA and a minor peak at the position of single stranded DNA. This result could be interpreted as the formation of interstrand crosslinks after treatment of DNA with 8-MOP and UV-A light. The results are in agreement with earlier findings of Hasan et al. (1991) who have reported nearly 74% crosslinked species in DNA treated with psoralen and UV-A light.

To detect interstrand crosslinks in poly(dA-dT)-8MOP photoadduct, it was heat denatured and passed through hydroxyapatite column (Fig. 13). The control experiment was carried out with native and denatured form of poly(dA-dT).poly(dA-dT). The elution of heat denatured photoadduct in the molarity regions of native conformer reiterate the diadduct nature of the photoadduct.

Analysis of Crosslinks by Bal 31 Digestion

To further probe the nature of adduct formed in native DNA irradiated with 8-MOP under UV-A light, the photoadduct was incubated with Bal 31 enzyme (Alteromonas espejiana) known to possess double stranded specific exonuclease activity.

The adduct incubated with Bal 31 for different time periods showed varying degree of digestion as revealed by progressively enhanced electrophoretic migration of

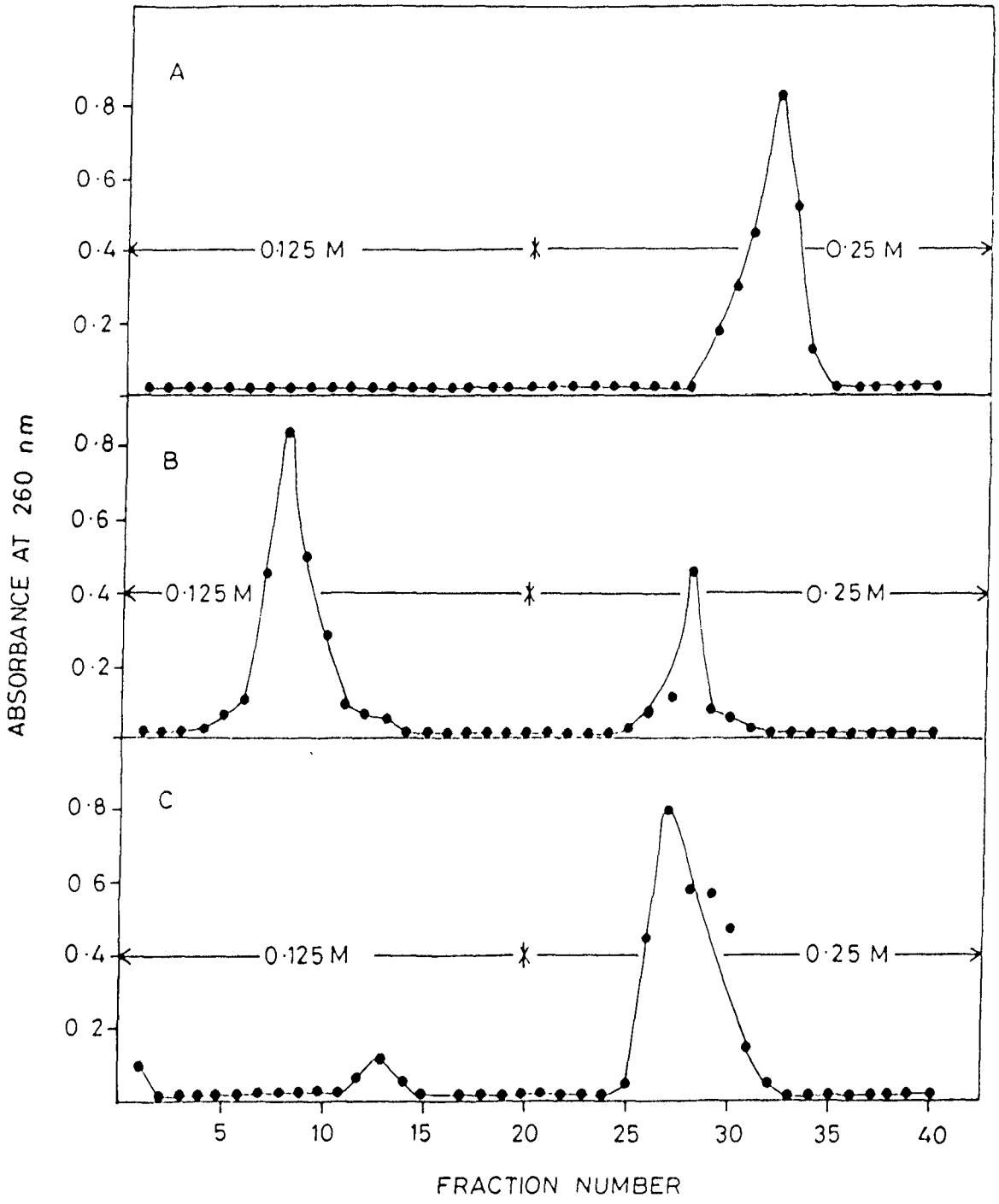


Fig.12. Hydroxyapatite column chromatography of (A) native DNA, (B) denatured DNA and (C) denatured DNA-8MOP photoadduct.

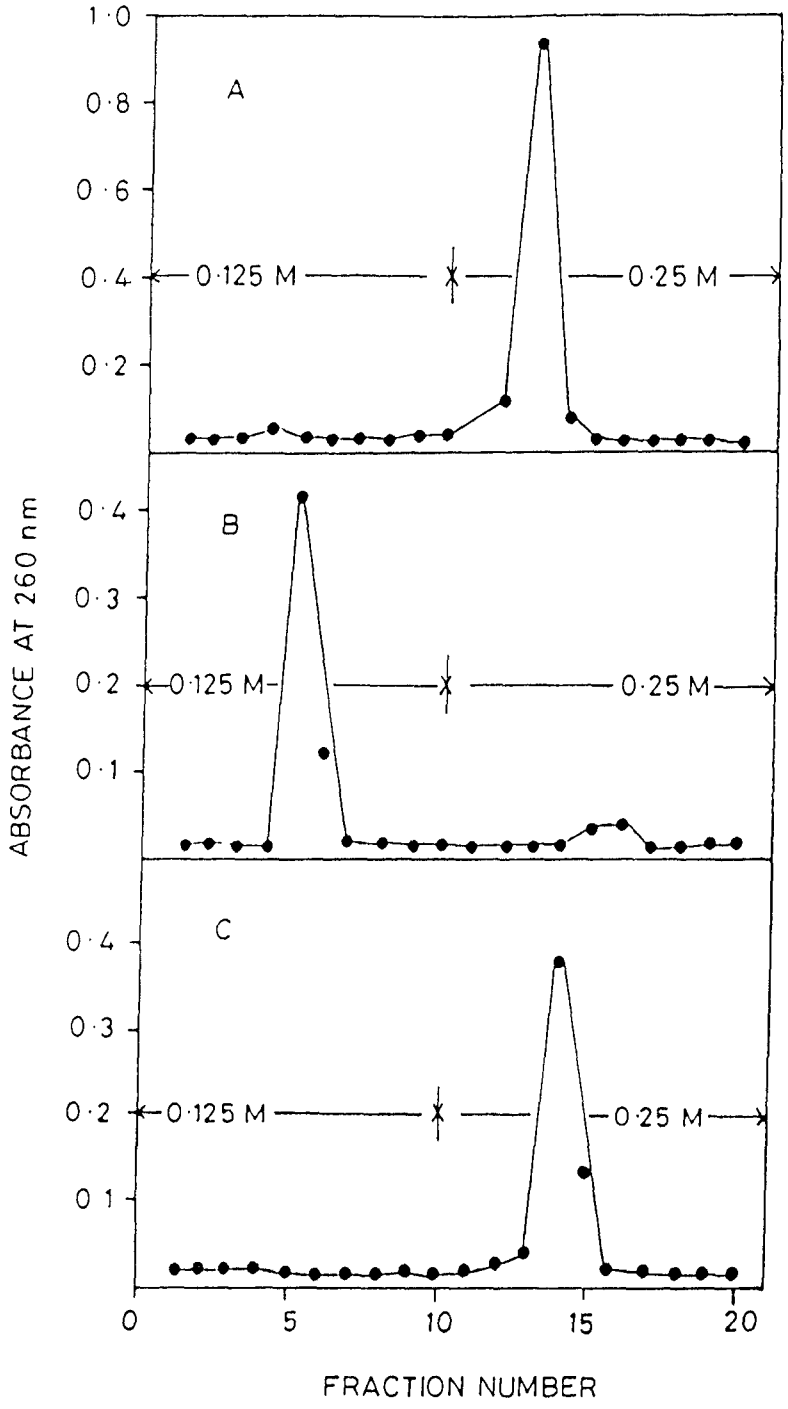


Fig.13. Hydroxyapatite column chromatography of (A) denatured poly(dA-dT)-8MOP photoadduct, (B) denatured poly(dA-dT).poly(dA-dT) and (C) native poly(dA-dT).poly(dA-dT).

residual polymer (Fig. 14). The photoadduct purified through hydroxyapatite (crosslinks) did not show any difference in its electrophoretic mobility for 0, 5, 10 and 20 min of enzyme incubation. By contrast, native DNA incubated for 20 min was almost completely digested. The formation of interstrand crosslink was confirmed by Bal 31 resistance, since this enzyme apparently identifies only crosslinks (Zhen et al., 1986). Analysis of results seems to indicate that the adduct obtained through hydroxyapatite was a pure crosslink.

Separation of Modified Bases

To determine the extent of modification in DNA bases as a consequence of photoadduct formation with 8-MOP, the modified DNA was hydrolyzed in presence of perchloric acid (60%) and treated at 100^{o}C for 1 hr to release the bases (Hasan and Ali, 1990). DEAE Sephadex A 50 chromatogram of modified DNA hydrolysate demonstrates the modification of thymine (Fig. 15). The extent of modification was calculated from the elution profile by measuring the peak area and was found to be 69 percent. The control experiment was carried out with hydrolyzed native DNA to locate the elution pattern of unmodified bases (Fig. 16). The individual bases were identified by their characteristic UV absorption pattern.

Induced Antibody Analysis by Immunological Techniques

The structural alteration in DNA as a result of photomodification with 8-MOP was analyzed by its potential to induce antibodies in experimental animals. The hydroxyapatite isolated crosslinks were complexed with MBSA in Freund's complete/incomplete adjuvant. The resulting complex was injected in rabbit and goat. Induced antibodies were analyzed by immunodiffusion, direct binding and competition-inhibition ELISA, quantitative precipitin titration, and gel retardation experiments.

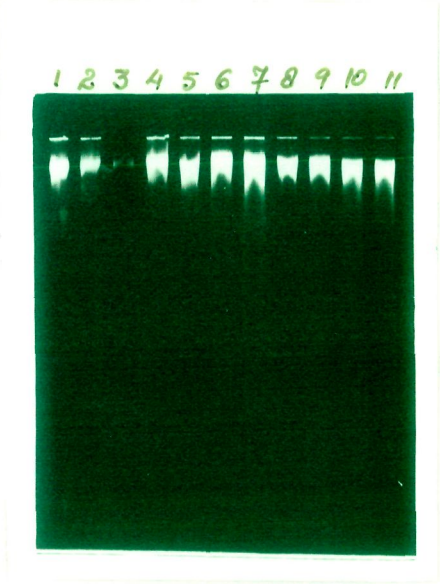


Fig. 14. Nuclease Bal 31 sensitivity of DNA-8MOP photoadduct. Lane 1, native DNA; lane 2, photoadduct and lane 3, native DNA exposed to Bal 31. Hydroxyapatite purified photocrosslink (lane 4-7) and non purified photoadduct (lane 8-11) was exposed to Bal 31 for 0,5, 10 and 20 min at 30°C and electrophoresed on 6% polyacrylamide gel.

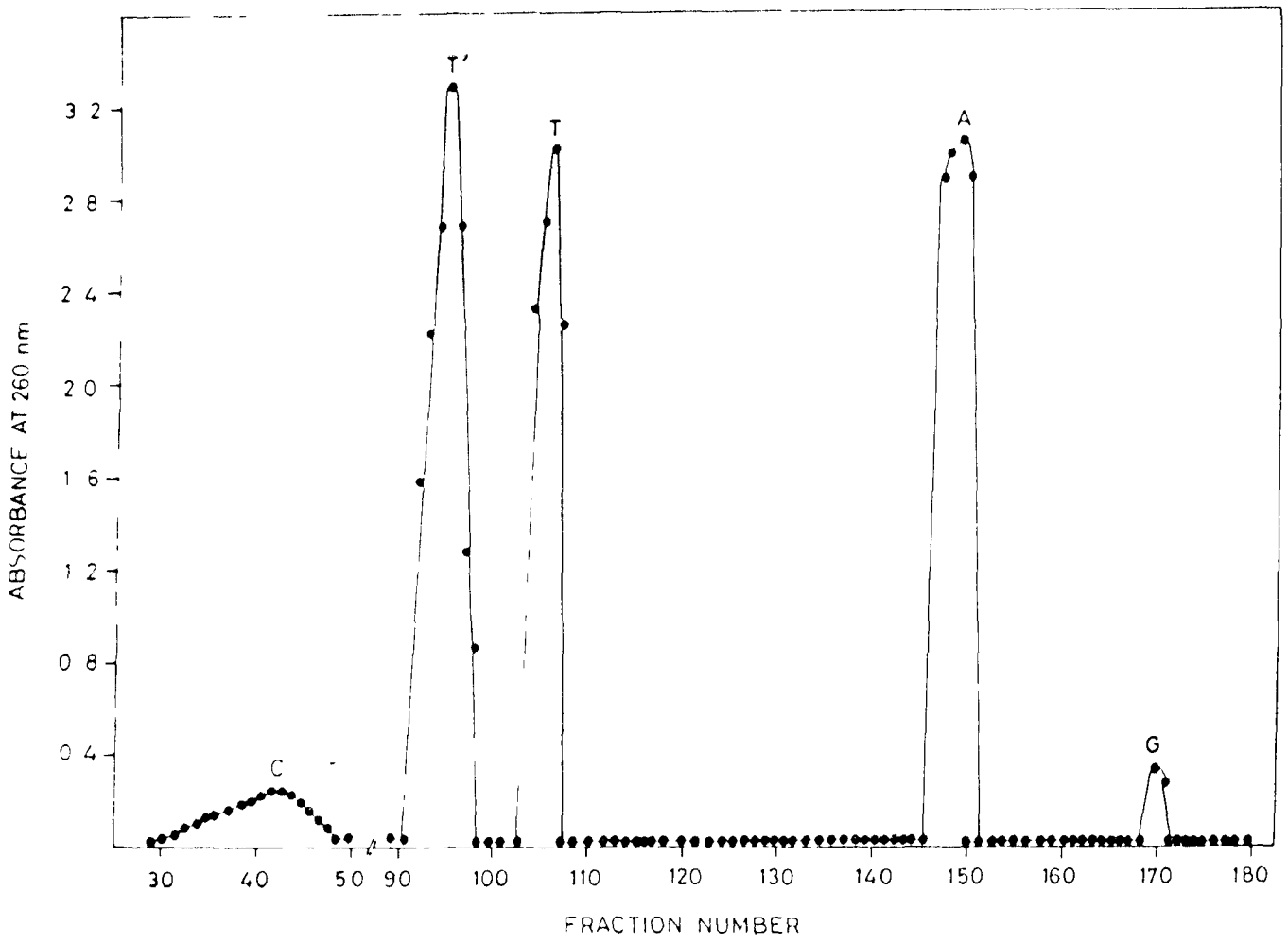


Fig.15. Elution profile of acid hydrolyzed DNA-8MOP photoadduct on DEAE Sephadex A 50 column.

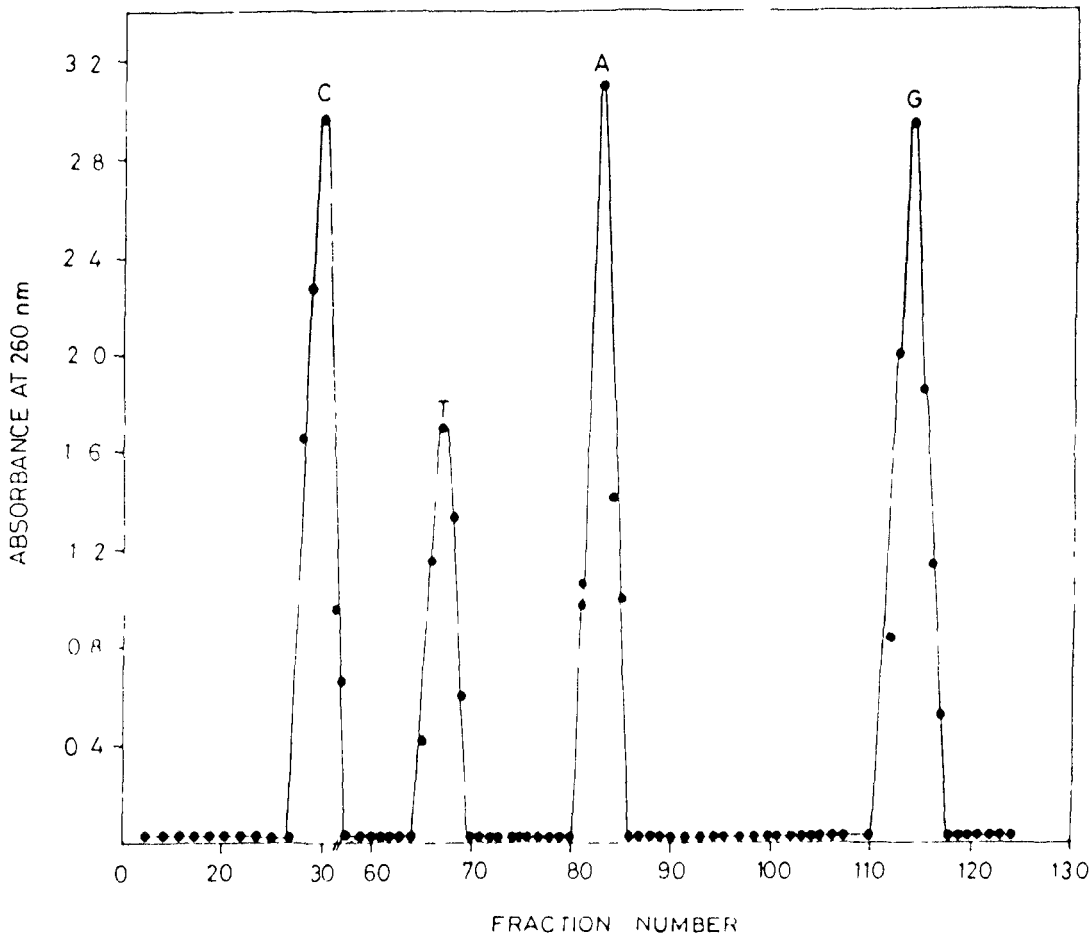


Fig.16. Acid hydrolyzed calf thymus DNA fractionated through DEAE Sephadex A 50 column.

The precipitating nature of induced antibodies against poly(dA-dT)-8MOP crosslink was analyzed by Ouchterlony immunodiffusion system. The titer of antibodies was found to be 1:16 (Fig. 17). Direct binding assay of induced antibodies on plate coated with immunogen revealed a titer of > 1:51200 (Fig. 18). Different dilutions of preimmune serum showed negligible binding with the immunogen. Similarly DNA-8MOP crosslink was highly immunogenic (Titer > 1:6400) in experimental animals (Fig. 19).

Isolation of IgG from Preimmune and Immune Sera

Immunoglobulin G (IgG) was isolated from immune and preimmune sera by DEAE Sephacel chromatography of 35% saturated ammonium sulphate precipitated γ-globulin fractions. Figures 20 and 21 show the elution of IgG by application of linear ionic strength gradient (0.01 M to 0.3 M Na-Pi, pH 8.0). The fractions were read at 280 nm and peak fractions of the chromatogram were pooled. The material was subjected to further purification on Sephadex G 200 column.

Exclusion Chromatography on Sephadex G 200 Column

To remove any low molecular weight contaminations from the isolated IgG, the pooled and dialyzed fractions were passed through previously equilibrated Sephadex G 200 column. Figures 22 & 23 show the elution profile of fractionated immune IgG. To analyze the purity of material, the absorbance was recorded at 251, 278 and 280 nm. The extent of IgG purity was checked by computing absorbance ratio (278/251) and was found to be 2.5, which indicate that the isolated IgG is sufficiently free of contamination by other proteins. It has been reported that for mammalian IgG the absorbance ratio (278/251) falls between 2.5-3.0 (Goding, 1976). The homogeneity of IgG was checked through SDS-PAGE. A single band movement

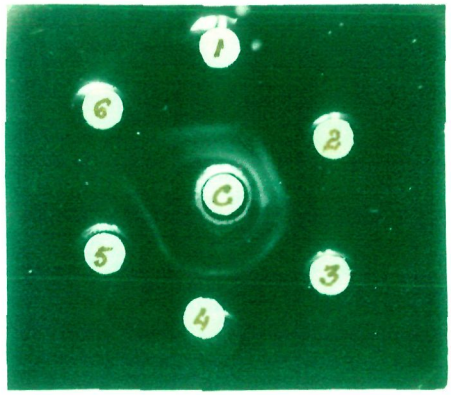


Fig. 17. Ouchterlony immunodiffusion of anti-poly(dA-dT)-8MOP antibodies. The central well (c) contained antigen and peripheral wells 1,2,3,4,5 and 6 represent neat, 1:2, 1:4, 1:8, 1:16 and 1:32 dilution of antiserum.

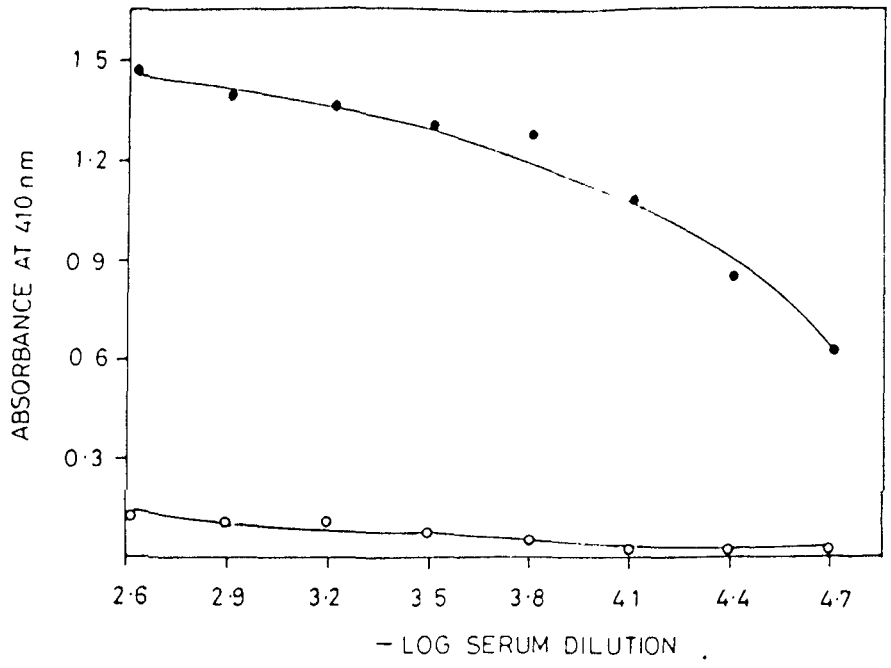


Fig.18. Direct binding ELISA of anti-poly(dA-dT)-8MOP antibodies. The microtiter plate was coated with poly(dA-dT)-8MOP photoadduct. Preimmune (—○—) and immune (—●—) sera.

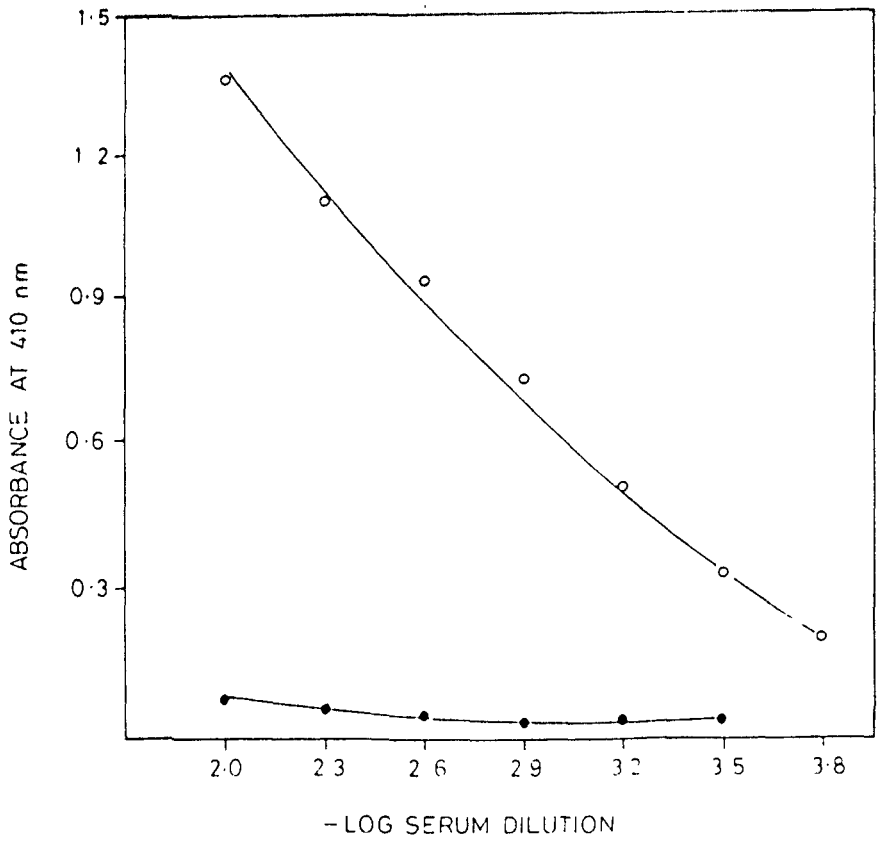


Fig.19. Assay of anti-DNA-8MOP antibody by ELISA. Microtiter plate was coated with DNA-8MOP photoadduct. Immune (—○—) and preimmune (—●—) sera .

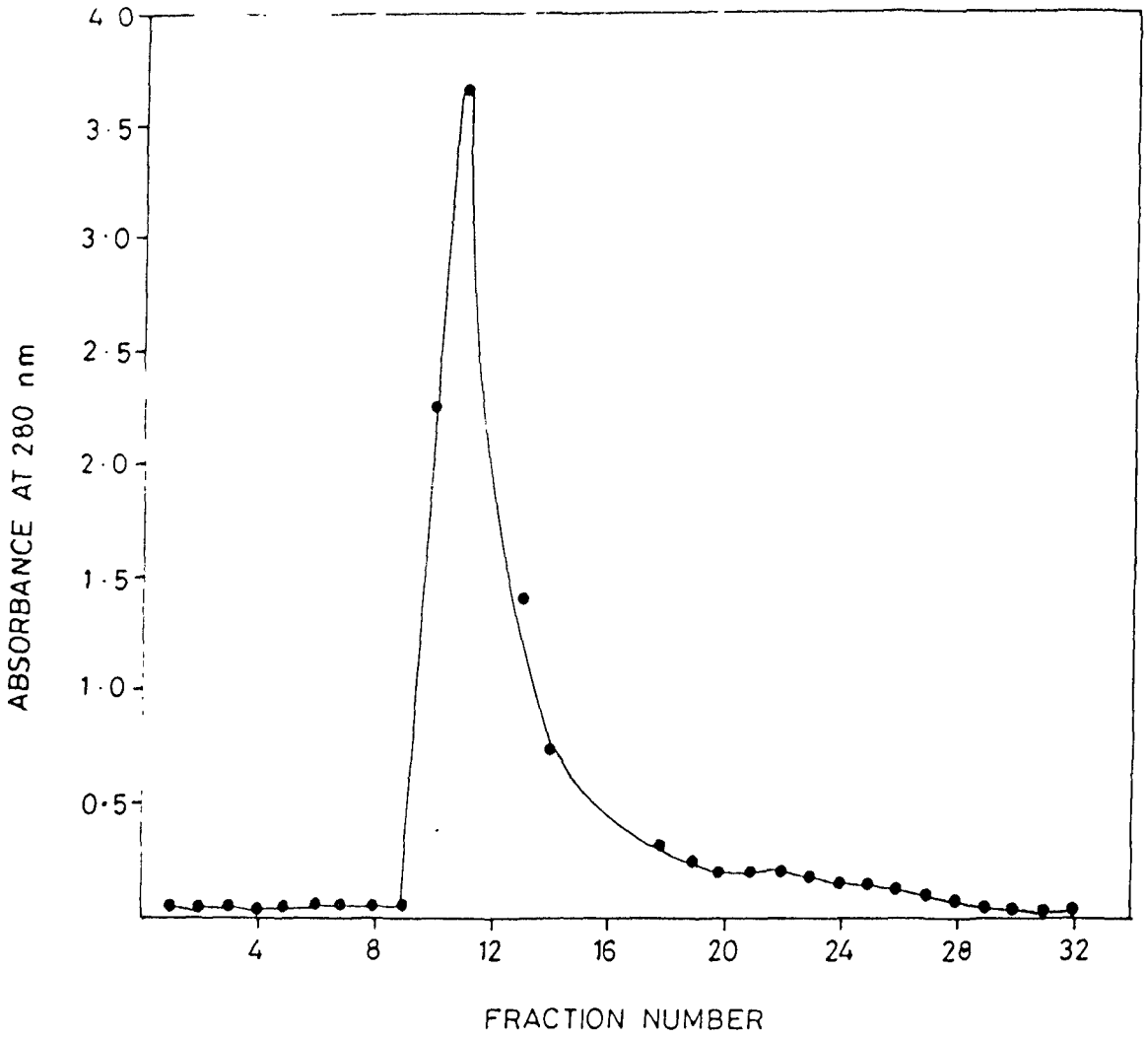


Fig.20. Isolation of anti-DNA-8MOP IgG on DEAE Sephacel column. The immune serum was precipitated with 35% saturated ammonium sulphate and dialyzed against 0.01 M Na-Pi, pH 8.0.

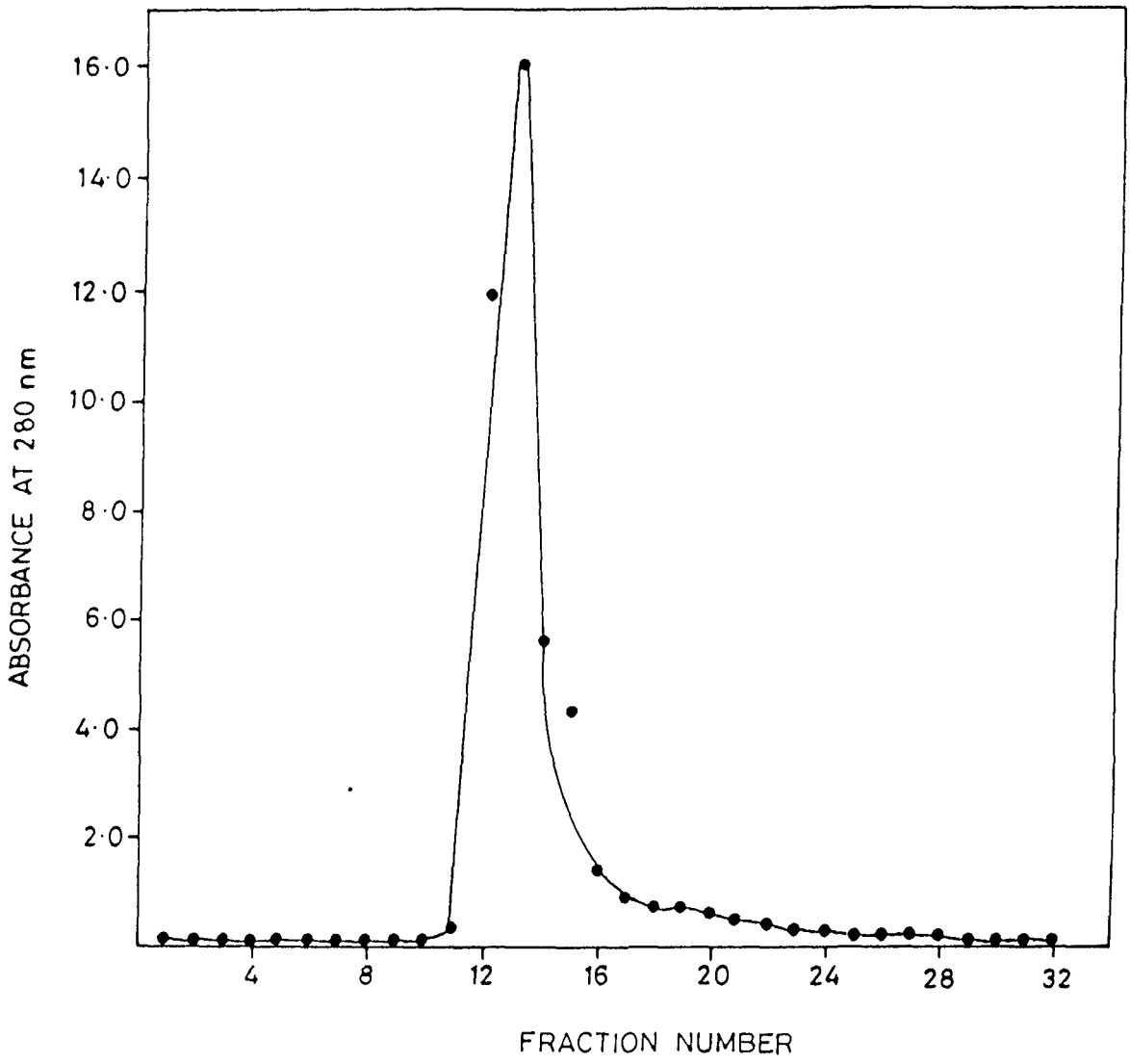


Fig.21. DEAE Sephacel column chromatography of anti-poly(dA-dT)-8MOP IgG.

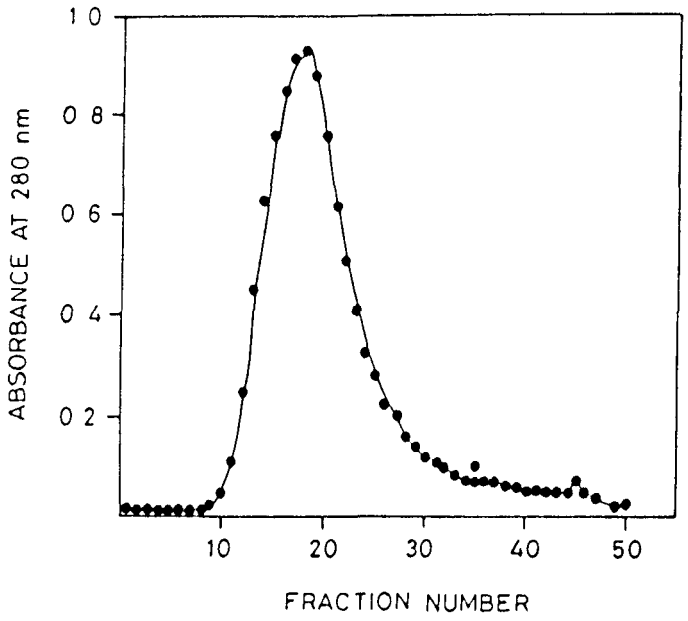


Fig.22. Exclusion chromatography on Sephadex G 200 column of DEAE Sephacel isolated anti-DNA-8MOP IgG.

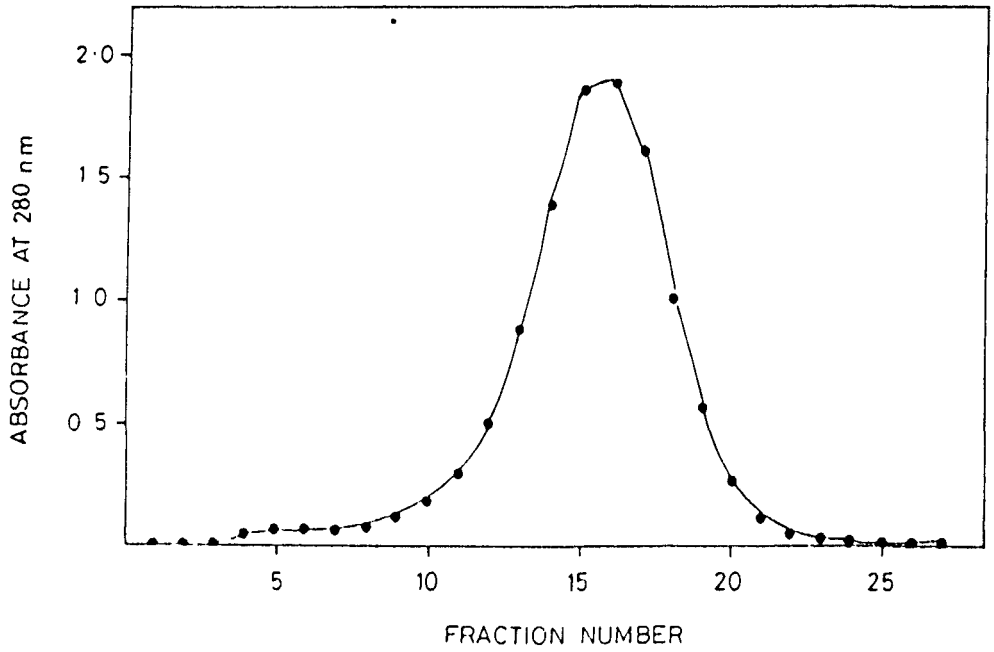


Fig.23. Purification of DEAE Sephacel isolated anti-poly(dA-dT)-8MOP IgG on Sephadex G 200 column.

reiterates the homogeneous nature of purified IgG (Figs. 24 & 25). Binding of isolated immune IgG towards respective immunogens was checked by direct binding ELISA (Figs. 26 & 27). The binding of preimmune IgG was of low magnitude.

Immunoaffinity Purification of Anti-DNA-8MOP Antibodies

The anti-DNA-8MOP IgG was subjected to further purification on polylysyl-Sepharose 4B matrix linked to DNA-8MOP photoadduct. The IgG was adsorbed to the column and washed extensively with PBS, pH 7.4 to remove unadsorbed molecules. The antibodies bound to DNA-8MOP-[polylysyl-Sepharose 4B] matrix were desorbed by stepwise application of increasing sodium chloride concentration. The fallthrough fractions were monitored at 280 nm and 260 nm (Fig. 28). The absorbance ratio (260/280) of peak I was found to be 0.54 while that of peak II the value was computed to be 1.8. These ratios are typical of IgG and DNA respectively. The protein peak was found to be reactive with anti-rabbit IgG (inset Fig. 28), whereas the material of peak II was nonreactive. Determination of protein by Coomassie Brilliant Blue and DNA with diphenylamine reagent indicated protein in peak I and DNA in peak II.

Binding Specificity of Induced Antibodies

The true recognition of DNA-8MOP crosslink (used as immunogen) by Sephadex G 200 purified anti-DNA-8MOP IgG was checked by inhibition ELISA. The immunogen binding to anti-DNA-8MOP IgG was evident from high degree of inhibition (Fig. 29). Fifty percent inhibition was seen at a photoadduct concentration of 0.16 ug/ml, which reiterates the specific immunogen-antibody interaction. When affinity purified antibodies were incubated with DNA-8MOP photoadduct and used in competitive binding assay, the percent inhibition increased from 86 to 99.2 percent (Fig. 30) and the concentration required for 50%

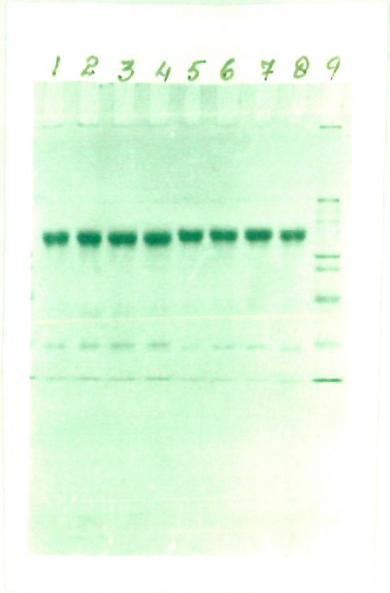


Fig.24

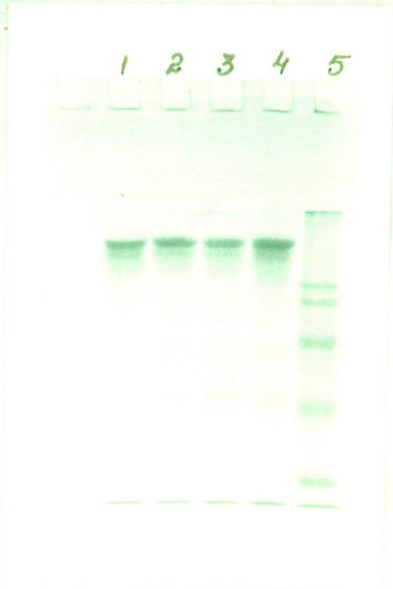


Fig.25

Fig.24. SDS-Polyacrylamide gel electrophoresis of Sephadex G 200 purified anti-DNA-8MOP IgG. Lanes (1-8) represent fractions 14-21. Lane 9 represent protein markers of known molecular weight.

Fig.25. Electrophoretic migration of Sephadex G 200 purified anti-poly(dA-dT)-8MOP IgG in polyacrylamide gel under denaturing conditions. Lanes (1-4) represent the fractions 14-17. Lane 5 is standard protein marker.

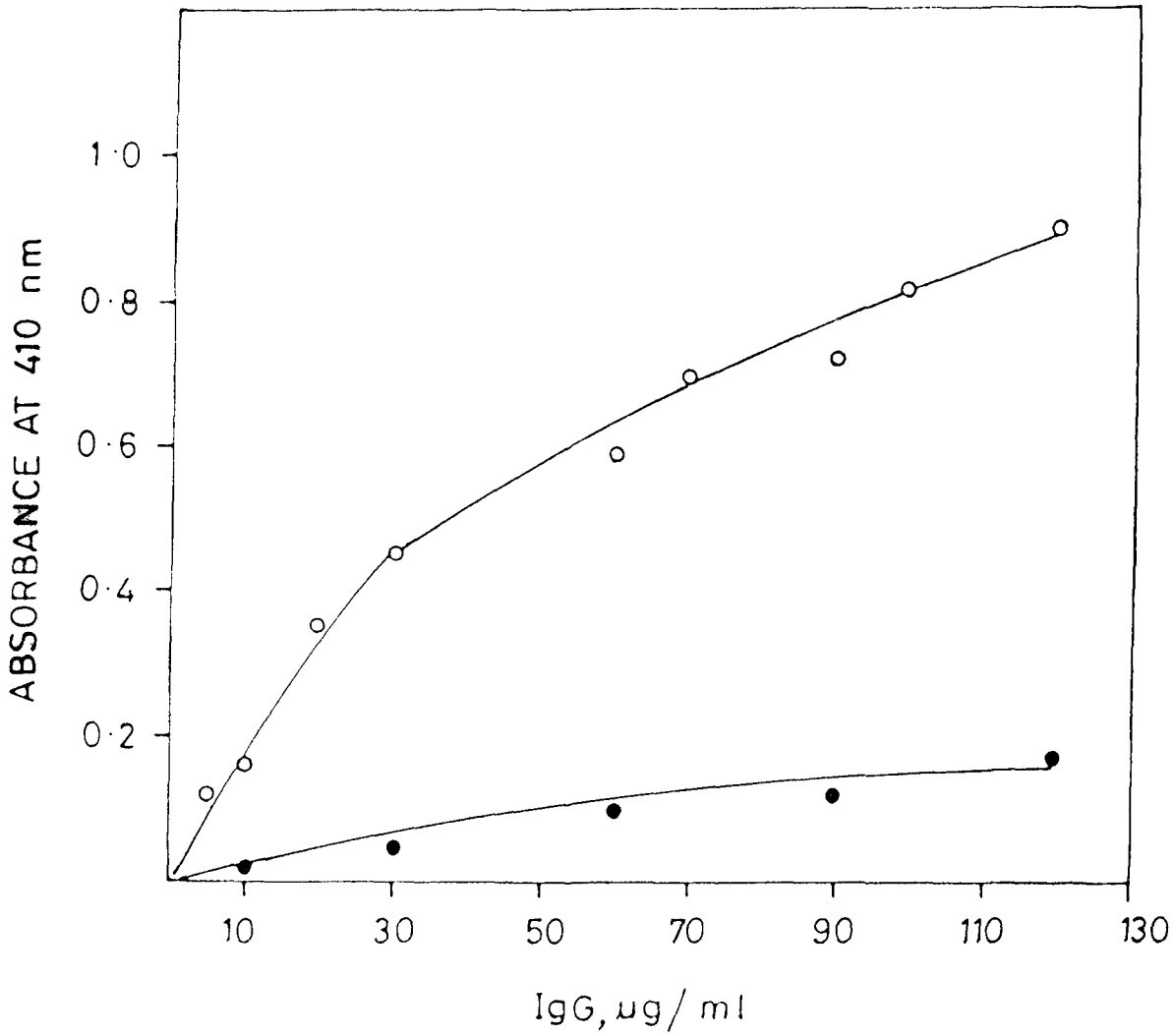


Fig.26. Direct binding ELISA of anti-DNA-8MOP IgG. The wells were coated with DNA-8MOP photocrosslink. Immune (—○—) and preimmune (—●—) IgG.

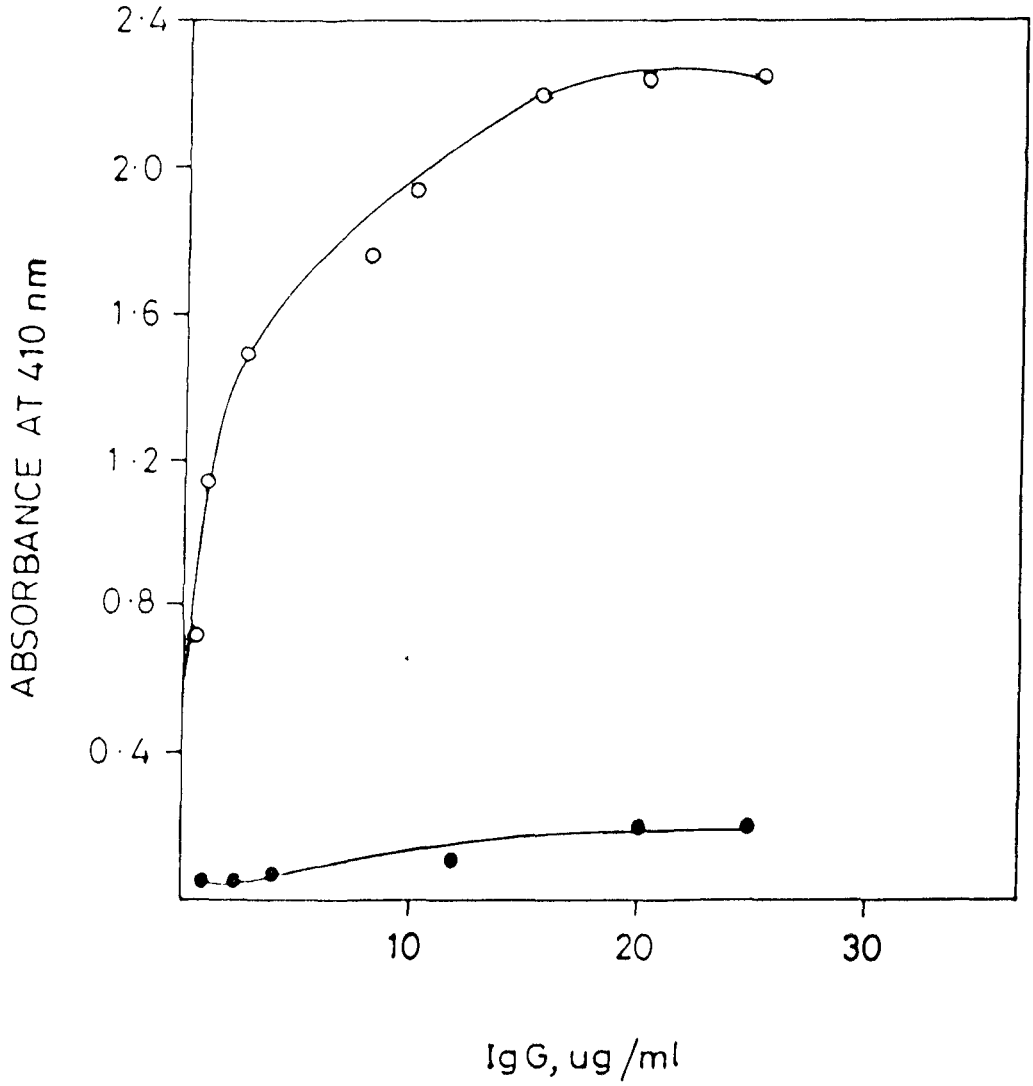


Fig.27. Assay of anti-poly(dA-dT)-8MOP IgG binding by ELISA. The plate was coated with poly(dA-dT)-8MOP crosslink. Immune (—○—) and preimmune (—●—) IgG.

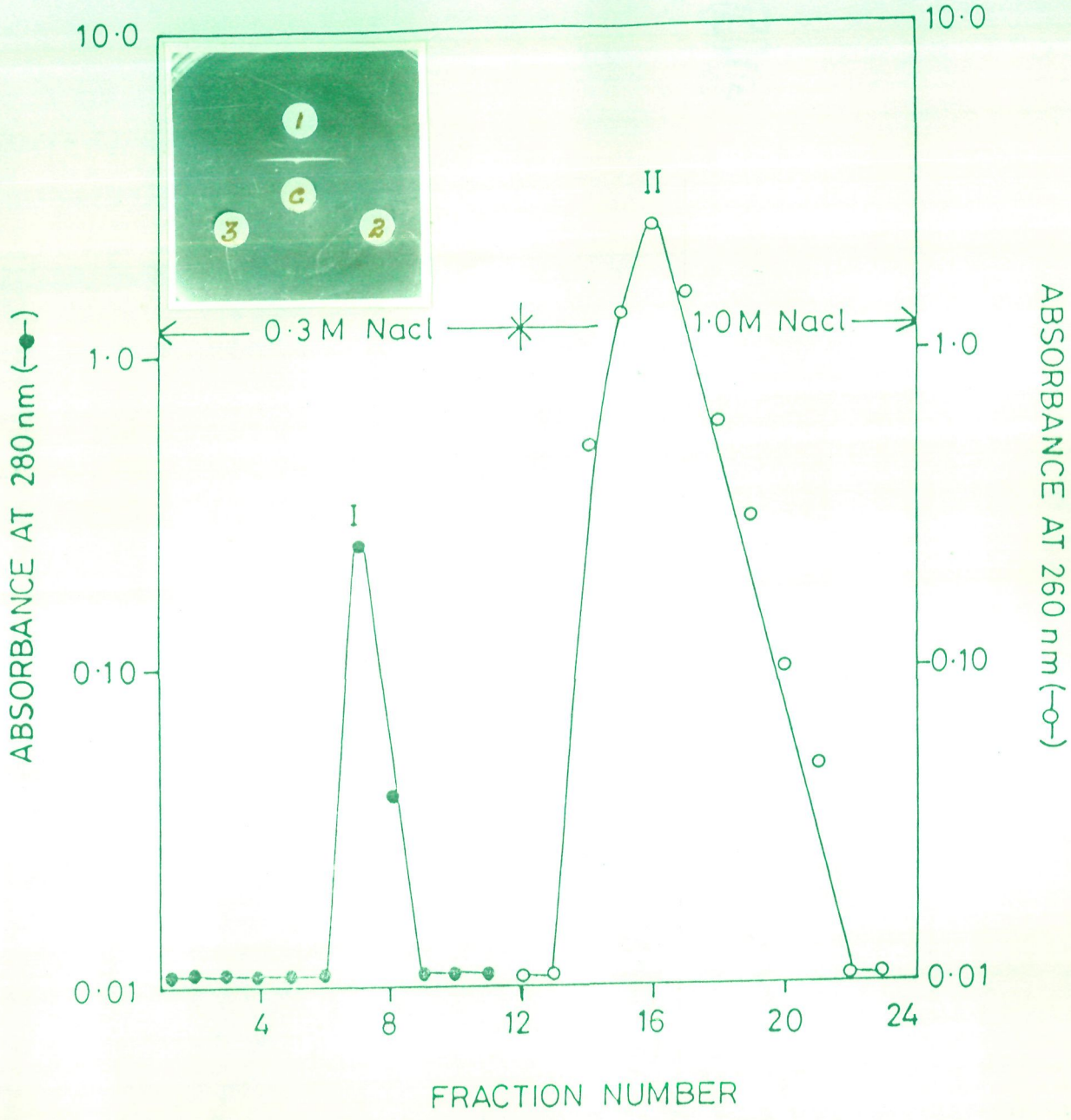


Fig.28. Immunoaffinity isolation of anti-DNA-8MOP antibodies on DNA-8MOP-[polylysyl-Sepharose 4B] column. The bound protein (peak I) and immobilized antigen (DNA-8MOP photoadduct) (peak II) was eluted by stepwise application of increasing molarity of sodium chloride solution. **Inset** shows the result of Ouchterlony immunodiffusion. The central well (c) contained anti-rabbit IgG, whereas peripheral wells 1 and 2 represent peak I and II respectively. Well (3) contained bovine serum albumin.

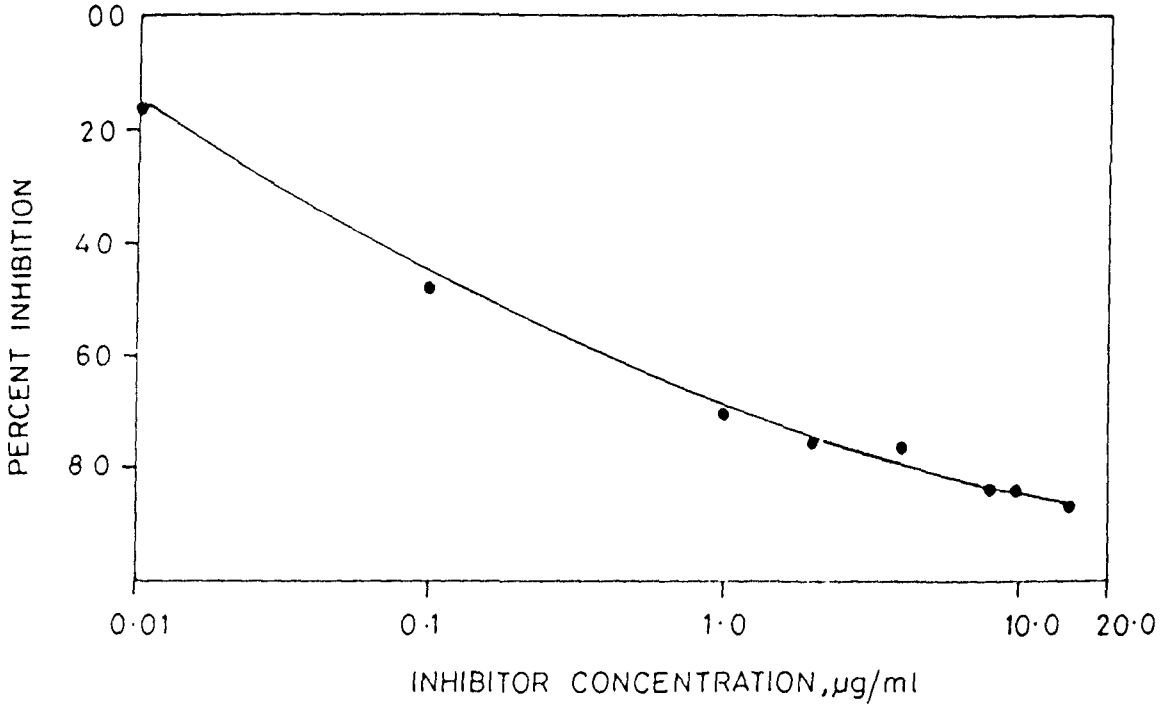


Fig.29. Inhibition of immunogen-antibody interaction by DNA-8MOP photoadduct.

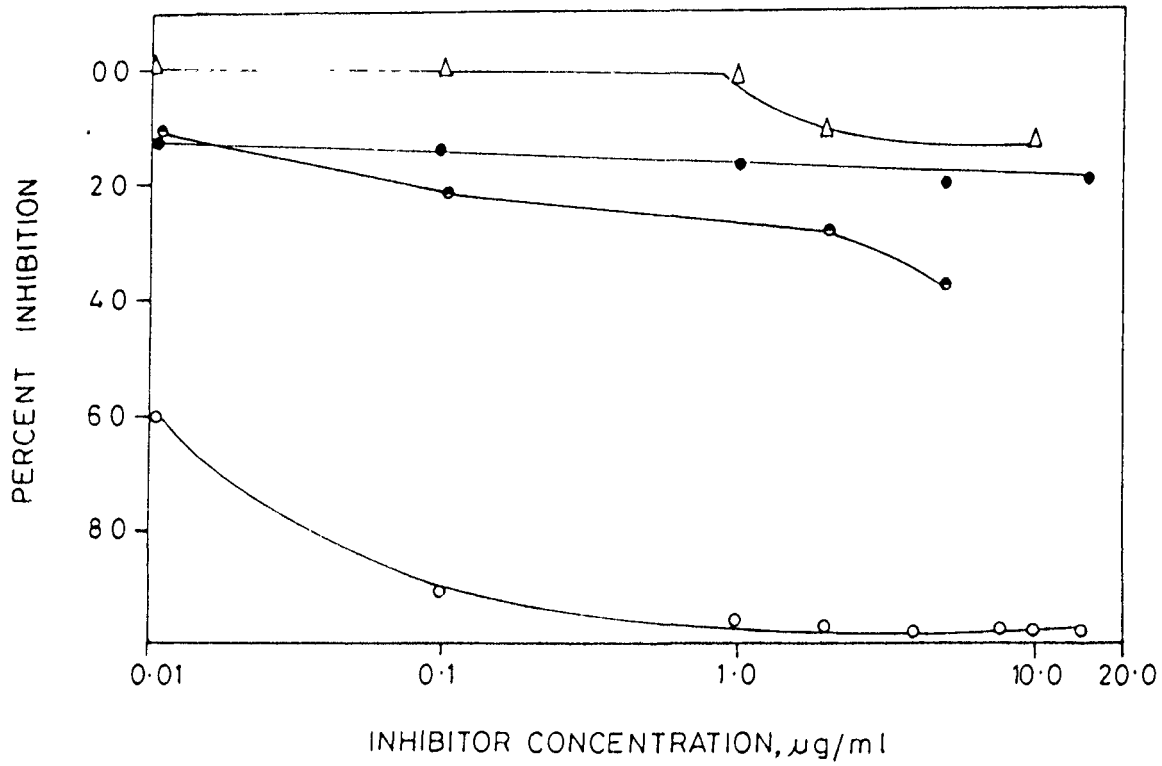


Fig.30.Competition ELISA of affinity isolated anti-DNA-8MOP IgG. The competitors were: DNA-8MOP photoadduct (-○-), native DNA (-●-), ssDNA (-◐-) and RNA (-△-).

elimination of antibody binding dropped from 0.16 ug/ml to < 0.01 ug/ml. The increased percent inhibition and lower competitor concentration required to achieve 50% inhibition shows specific epitope recognition on DNA-8MOP crosslink by affinity purified IgG.

An attempt was made to ascertain whether antibodies (affinity purified) were mainly confined to the conformation of immunogen or were also recognizing the polyanionic backbone and bases therein. Nucleic acid polymers were also employed as competitors of antibody-immunogen complex. As evident from the inhibition data (Table 7), the antibodies showed some cross reactivity with heat denatured DNA. Almost negligible inhibition was obtained with native DNA and buffalo thymus RNA (Fig. 30).

Quantitative Precipitin Titration

The immunogen-antibody interaction was also characterized by quantitative precipitin titration. Varying amounts of DNA-8MOP photoadduct (0-50 ug) was mixed with 120 ug each of immune and preimmune IgG and incubated for 2 hr at 37^{o}C and overnight at 4^{o}C. The result obtained were typical of precipitin titration curve (Fig. 31). Maximum antibody binding occurred at a photoadduct concentration of 25.0 ug. Protein analysis of immune complex showed that 103 ug of added immune IgG was bound to the photoadduct. Further analysis of precipitin data revealed that there were 4.1 ug of immune IgG/ug of DNA-8MOP photoadduct. The affinity constant of anti-DNA-8MOP antibodies was evaluated by Scatchard analysis (Fig. 32) and apparent association constant was computed to be 1.13 x 10^{-9} M.

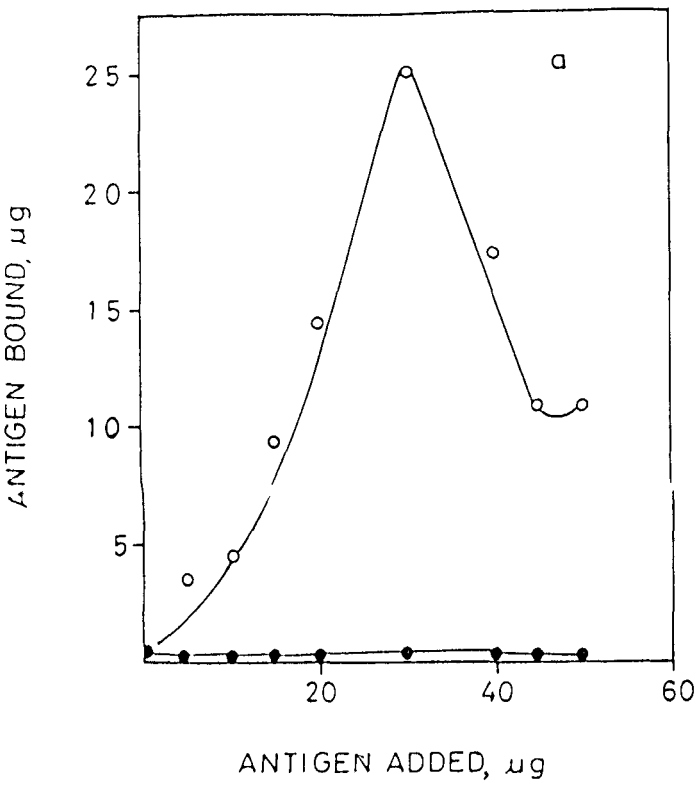
Binding Characteristics of Anti-poly(dA-dT)-8MOP Antibodies

The antibodies against poly(dA-dT)-8MOP crosslink

TABLE 7

Competition-inhibition data of affinity purified anti-DNA-8MOP IgG

Competitor	Inhibitor concentration (ug/ml)	Maximum percent inhibition
DNA-8MOP	10.0	99.2
ssDNA	5.0	38.5
Native DNA	15.0	19.0
RNA	10.0	13.0



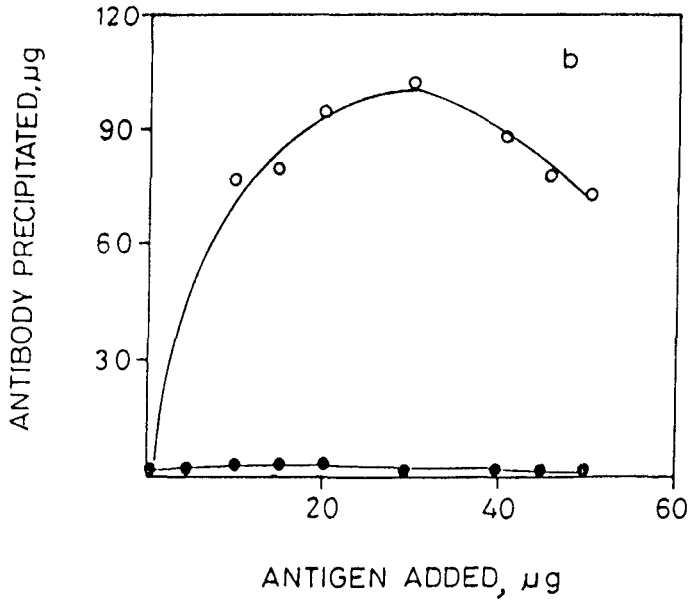


Fig.31. Binding characteristics of anti-DNA-8MOP antibodies. Amount of antigen bound (a) and amount of antibody precipitated (b). The amount of immune IgG was kept constant at 120 ug in a total volume of 0.2 ml. Immune (-○-) and preimmune (-●-) IgG.

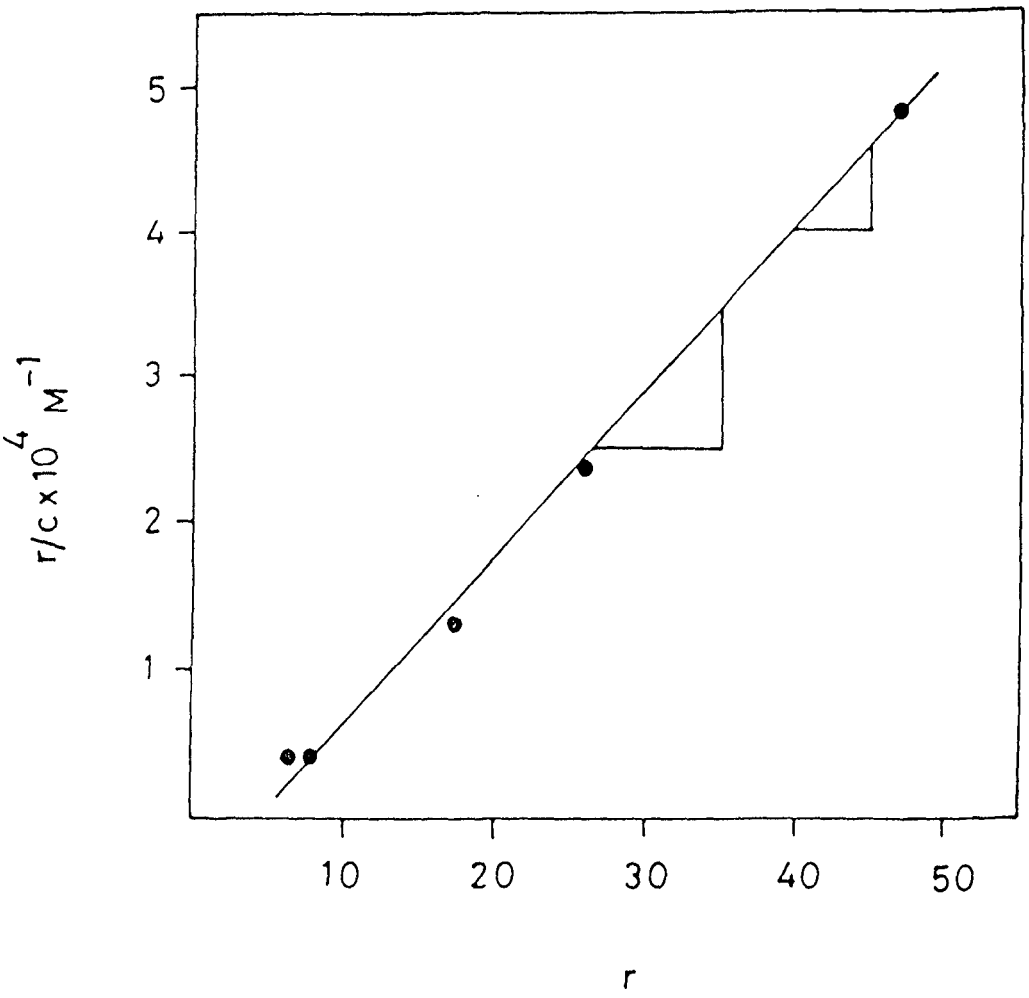


Fig.32.Determination of anti-DNA-8MOP antibody affinity by Scatchard plot.

were found to be highly specific as revealed by competitive binding assay (Fig. 33). The antibody-immunogen interaction was eliminated to the level of 90% when immunogen was used as competitor of antibody binding. Low level of inhibition was observed with DNA-psoralen and DNA-8MOP photoadducts.

Antibody binding to immunogen was also analyzed in agarose gel. Mixtures of poly(dA-dT)-8MOP photoadduct and varying amounts of induced antibodies were subjected to agarose gel electrophoresis. The electrophoretic migration of immune complex(es) was progressively retarded (Fig. 34). With increase in antibody concentration the amount of immune complex formed was increased as indicated by the enhanced fluorescence intensity at the top of the well. Whereas unbound poly(dA-dT)-8MOP showed proportional decrease in their fluorescence. Preimmune goat IgG included as negative control showed no immune complex formation. The antibody binding to DNA-8MOP and DNA-psoralen crosslinks were also evident from retarded electrophoretic migration of immune complex compared to fast migrating antigen (Fig. 35).

Native DNA brominated under high salt (4M NaCl) has been shown to present Z- or Z-like epitope (Hasan and Ali, 1990). When brominated DNA was incubated with anti-poly(dA-dT)-8MOP antibodies and used in competition ELISA, the antibody-immunogen binding was eliminated to the level of 51.3 percent (Fig. 33). The binding of induced antibodies to brominated DNA was also evident from band shift assay (Fig. 35).

To check whether anti-poly(dA-dT)-8MOP antibodies were polyspecific, competitive binding assay was carried out. Native DNA, poly(rG).poly(dC) [known to present features of A- or A-like DNA], poly(dG).poly(dC), ROS-DNA, DNA-lysine photoadduct, poly(dA-dT).poly(dA-dT),

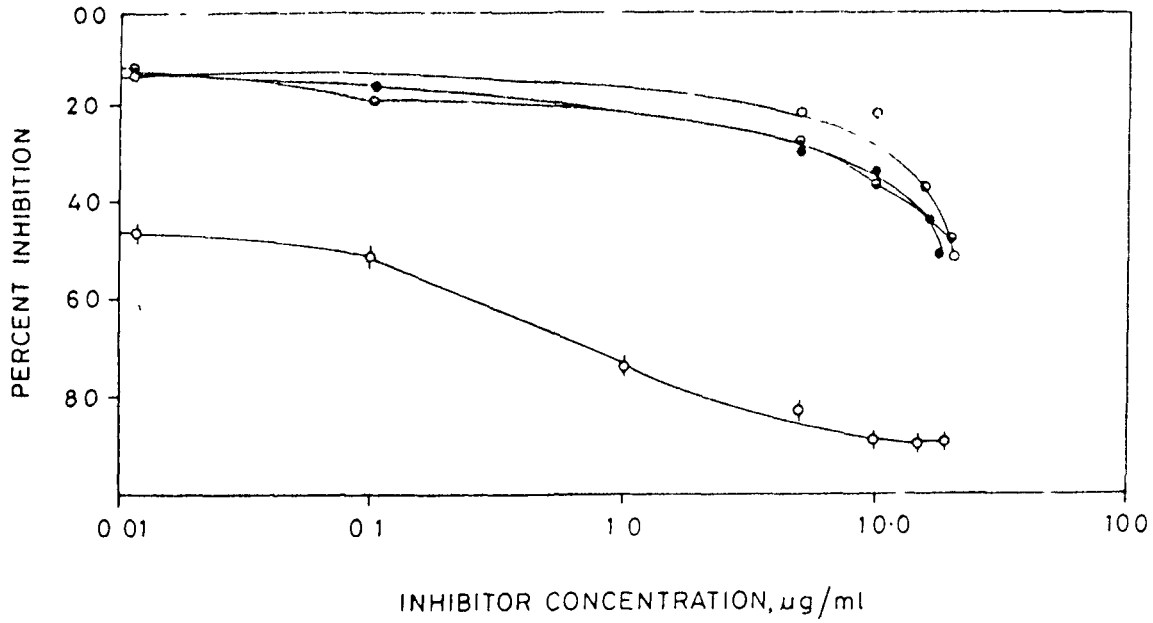


Fig.33. Inhibition of anti-poly(dA-dT)-8MOP IgG binding to poly(dA-dT)-8MOP photoadduct. The competitors were: poly(dA-dT)-8MOP (-○-), DNA-8MOP (-○-), DNA-psoralen (-●-) and brominated DNA (-●-).

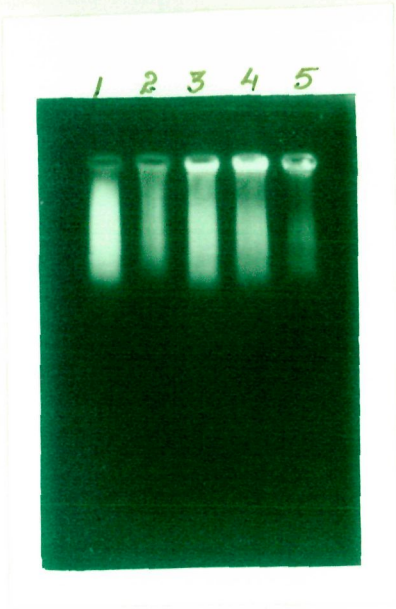


Fig.34. Binding of anti-poly(dA-dT)-8MOP IgG to poly(dA-dT)-8MOP photoadduct as analyzed by gel retardation assay. Poly(dA-dT)-8MOP photoadduct (0.5 ug) (lane 1) was incubated with 30 ug of preimmune goat IgG (lane 2) and different amounts of induced antibodies (10 ug-lane 3; 20 ug-lane 4 and 30 ug-lane 5) for 2 hr at 37°C and overnight at 4°C. The samples were run on 1% agarose gel for 2 hr at 40 volts.

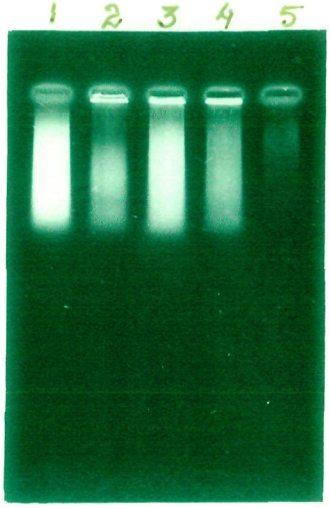


Fig.35. Gel retardation assay of anti-poly(dA-dT)-8MOP antibodies binding with various modified nucleic acid polymers. Thirty microgram of anti-poly(dA-dT)-8MOP antibody was incubated with 0.5 ug each of poly(dA-dT)-8MOP (lane 2), DNA-psoralen (lane 3), DNA-8MOP (lane 4) and brominated DNA (lane 5) for 2 hr at 37°C and overnight at 4°C. Lane 1 shows the migration of poly(dA-dT)-8MOP photoadduct (0.5 ug) incubated with buffer alone. Electrophoresis was carried out on 1% agarose gel for 2 hr at 40 volts.

buffalo thymus RNA and heat denatured DNA were used as competitors of antibody binding. The level of inhibition in immunogen-antibody interaction by these competitors are presented in Table 8.

Native DNA and poly(rG).poly(dC) incubated with anti-poly(dA-dT)-8MOP IgG did not show any retardation in electrophoretic migration in agarose gel (Fig. 36) which further confirms that the induced antibodies were mainly directed to the modified epitopes of poly(dA-dT)-8MOP photoadduct.

Quantitative Precipitin Titration

The specificity of anti-poly(dA-dT)-8MOP antibodies for the immunogen was also analyzed by incubating 100 ug of antibodies with varying amount (0-25 ug) of poly(dA-dT)-8MOP photoadduct. The complex was incubated for 2 hr at 37^{o}C and overnight at 4^{o}C to achieve equilibrium of antigen-antibody interaction. The analysis of precipitin data resulted in the appearance of typical precipitin curve (Fig. 37). Fifty percent of added antibodies were bound by 3.25 ug of immunogen. The maximum amount of antibody binding was seen at an antigen concentration of 10 ug. The binding data was analyzed and antibody affinity was calculated by Langmuir isotherm plot (Fig. 38). The apparent association constant was computed to be 6.80 x 10^{-10}M.

Anti-ZDNA Antibody Binding to Poly(dA-dT)-8MOP and DNA-8MOP Crosslinks

The neoepitopes on poly(dA-dT)-8MOP and DNA-8MOP crosslink were identified by previously defined monoclonal anti-ZDNA antibody (Z22). The specificity of Z22 for typical Z-epitope was evident from 78% inhibition of its binding to Z-DNA at 10 ug/ml when same was used as competitor (Fig. 39). Fifty percent inhibition was seen at 2.2 ug/ml of Z-DNA. This demonstrates that Z22 is

TABLE 8

Competitive binding data of anti-poly(dA-dT)-8MOP IgG

Competitor	Competitor concentration for 50% inhibition (ug/ml)	Maximum percent inhibition
Poly(dA-dT)-8MOP	0.08	90.0
Poly(dA-dT)	*	15.0
Brominated DNA	18.0	51.3
DNA-8MOP	19.0	54.4
DNA-psoralen	*	48.6
Native DNA	*	31.3
RNA	*	34.6
ssDNA	*	NI
Poly(rG).poly(dC)	*	30.0
Poly(dG).poly(dC)	*	22.1
DNA-lysine photoadduct	*	NI
ROS-DNA	*	NI

* 50% inhibition was not obtained

NI = No inhibition

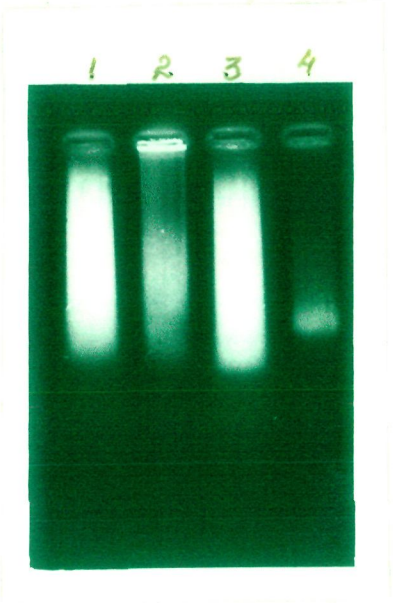
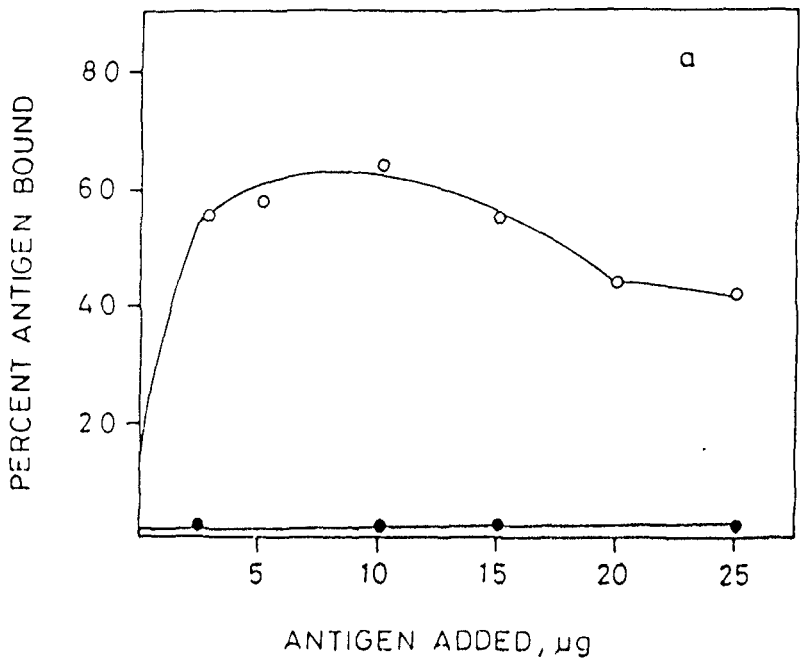


Fig.36. Binding of anti-poly(dA-dT)-8MOP IgG with nucleic acid polymers as analyzed by gel retardation assay. Thirty microgram of anti-poly(dA-dT)-8MOP antibody was incubated with 0.5 ug each of poly(dA-dT)-8MOP (lane 2), nDNA (lane 3) and poly(rG).poly(dC) (lane 4) for 2 hr at 37°C and overnight at 4°C. Lane 1 depicts the migration of poly(dA-dT)-8MOP photoadduct (0.5 ug) incubated with buffer alone. The samples were electrophoresed on 1% agarose gel.



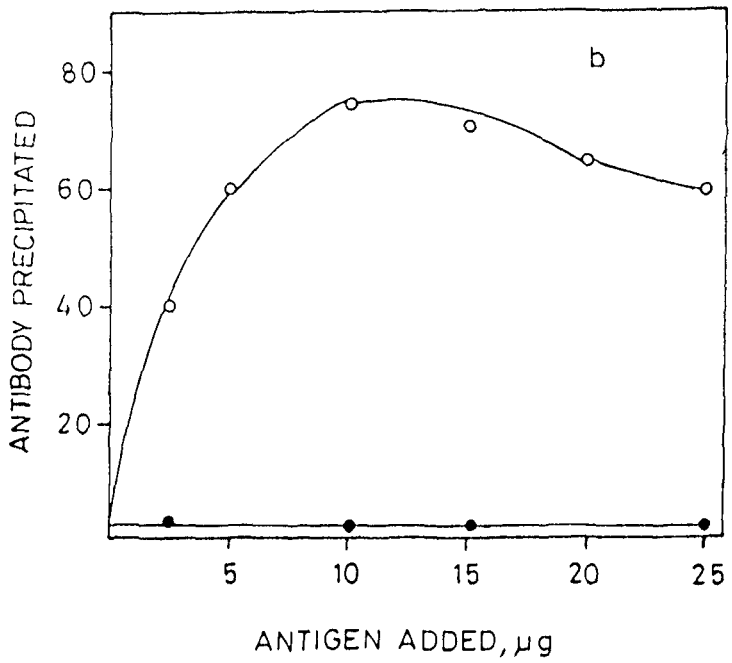


Fig.37. Quantitative binding curves illustrating the effect of antibody and immunogen concentration on binding data. (a) Shows the percent antigen bound by immune (—○—) and preimmune (—●—) IgG as a function of poly(dA-dT)-8MOP concentration. (b) Shows the amount of immune (—○—) and preimmune (—●—) IgG bound as a function of poly(dA-dT)-8MOP concentration.

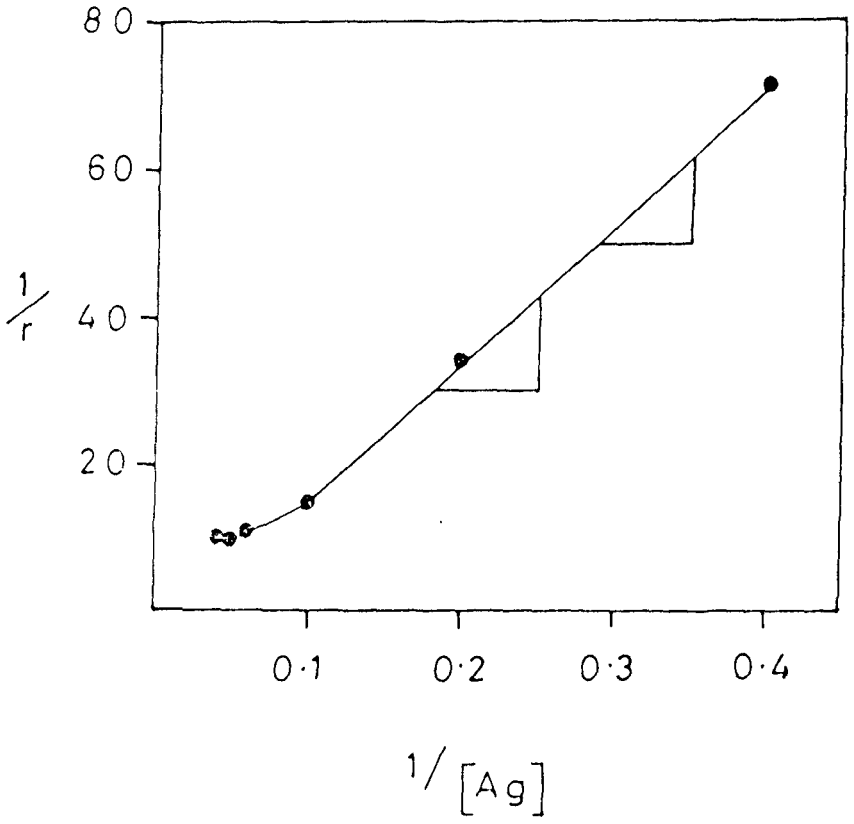


Fig.38. Evaluation of anti-poly(dA-dT)-8MOP IgG affinity by Langmuir isotherm plot.

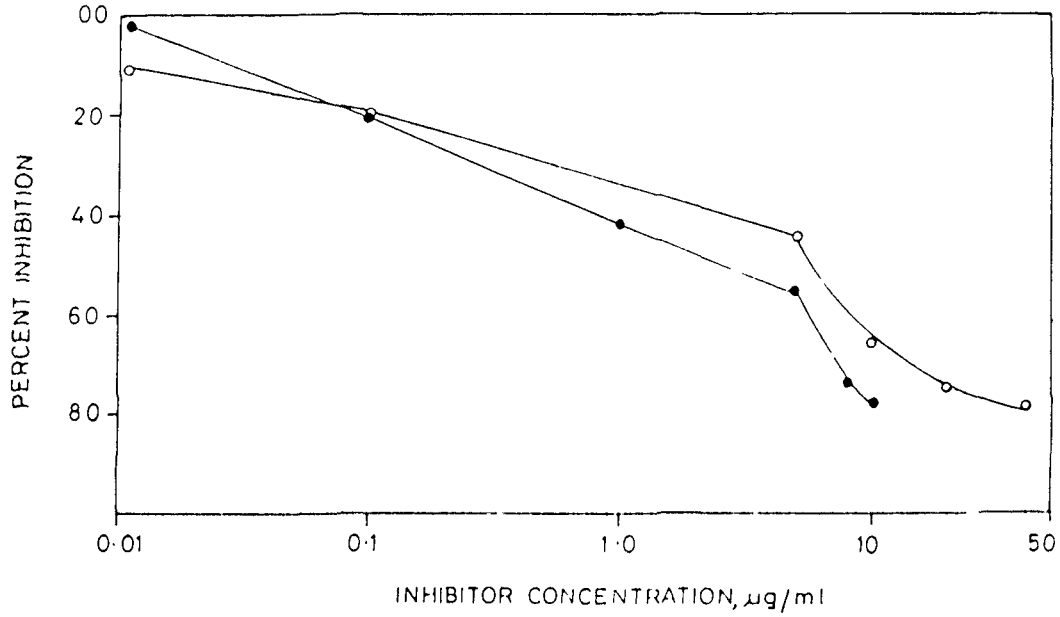


Fig.39. Inhibition of anti-ZDNA antibody (Z22) binding to Z-DNA by poly(dA-dT)-8MOP photoadduct (—○—) and Z-DNA (—●—).

highly specific probe for Z-DNA conformation. Therefore, monoclonal Z22 antibody was employed as valuable reagent to locate or identify the presence of Z- or Z-like epitopes on DNA-8MOP and poly(dA-dT)-8MOP photocrosslinks. The high specificity of Z22 antibodies for poly(dA-dT)-8MOP crosslink could be perceived from 79% inhibition in its activity at 40 ug/ml (Fig. 39). Fifty percent inhibition was obtained at 6.0 ug/ml of poly(dA-dT)-8MOP photoadduct. Similar binding pattern was obtained with DNA-8MOP photoadduct. A maximum of 78.2% inhibition in the Z22 antibody binding was obtained at 40 ug/ml of DNA-8MOP photoadduct (Fig. 40). Fifty percent inhibition in this case was recorded at a much higher inhibitor concentration (27 ug/ml). On the basis of fifty percent inhibition data (Table 9) it could be observed that poly(dA-dT)-8MOP photoadduct possessed atleast 4.5 times higher affinity towards monoclonal Z22 IgG compared to DNA-8MOP photoadduct. The binding of Z22 antibody with photoadducts was also studied by gel retardation assay. The Z22 antibody (0-28 ug) was incubated with 0.5 ug of either of the photoadducts for 2 hr at 37^{o}C and overnight at 4^{o}C. The immune complex thus formed was analyzed in agarose gel (Figs. 41 & 42). The fluorescence intensity of retarded bands increases with increase in Z22 concentration whereas unbound antigen showed proportional decrease in fluorescence intensity. Gel retardation and competitive binding assay results reiterate the epitope sharing between prototype Z-DNA and poly(dA-dT)-8MOP and DNA-8MOP photocrosslinks.

Human Anti-DNA Autoantibodies Binding to Photoadducts

Anti-DNA antibodies derived from human lupus have demonstrated polyreactivity with respect to multiple DNA antigens. Besides nDNA (in typical B-conformation), these antibodies have exhibited strong binding with A- and Z- conformation of DNA (Alam and Ali, 1992). Since both poly(dA-dT)-8MOP and DNA-8MOP photoadducts have been

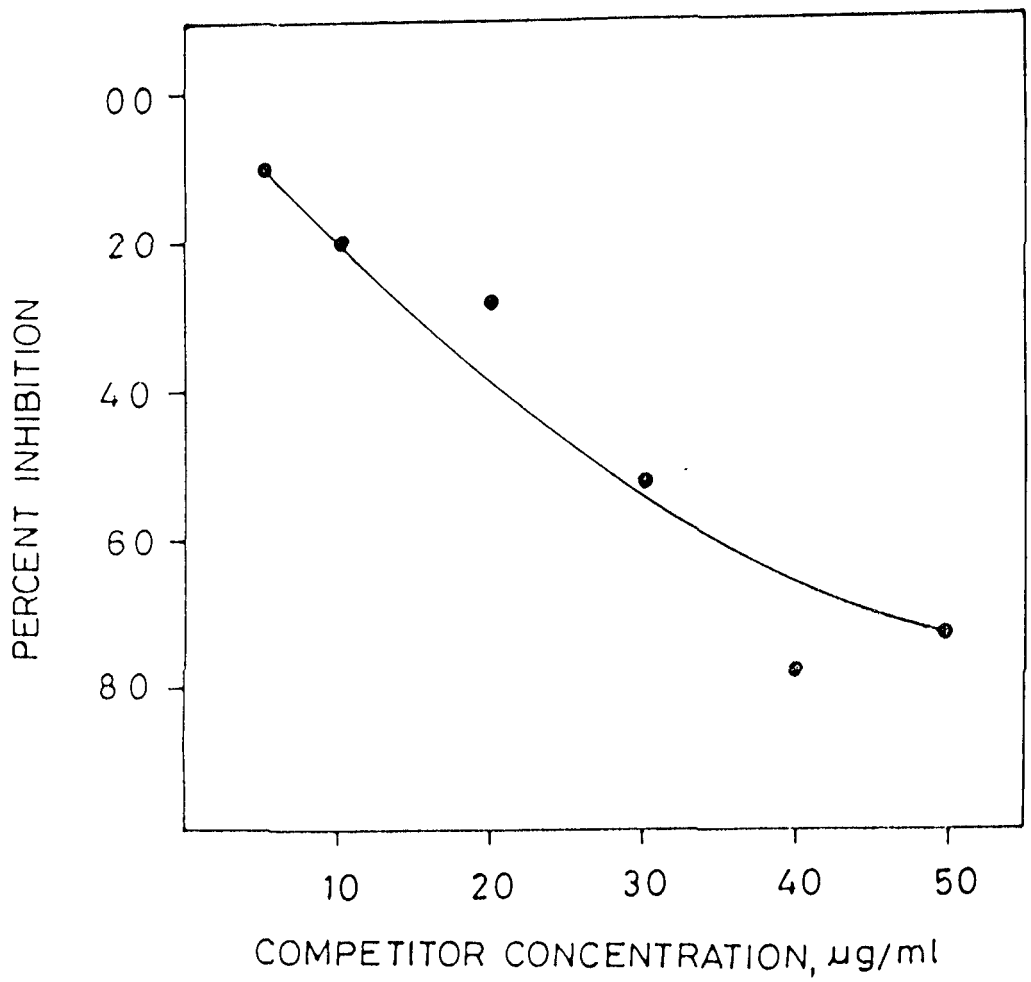


Fig.40. Inhibition of Z22 antibody binding to Z-DNA by DNA-8MOP photoadduct.

TABLE 9

Binding parameters of Z22 antibody with Z-DNA and nucleic acid-8MOP photoadducts

Inhibitor	Inhibitor concentration for 50% inhibition (ug/ml)	Maximum inhibitor concentration (ug/ml)	Maximum percent inhibition
Z-DNA	2.2	10	78
Poly(dA-dT)-8MOP	6.0	40	79
DNA-8MOP	27.0	40	78.2

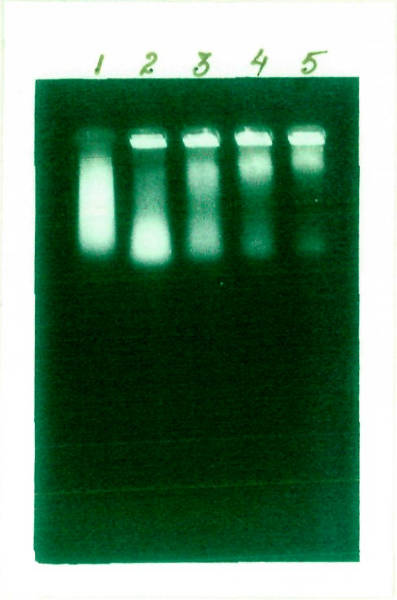


Fig.41. Binding of poly(dA-dT)-8MOP photoadduct by Z22 IgG as analyzed by gel retardation assay. Poly(dA-dT)-8MOP photoadduct (0.5 ug) was incubated with buffer (lane 1) and increasing concentrations of anti-ZDNA (Z22) IgG (4 ug - lane 2; 8 ug-lane 3; 16 ug-lane 4 and 28 ug-lane 5) for 2 hr at 37°C and overnight at 4°C. the samples were run on 1% agarose gel for 2 hr at 40 volts.

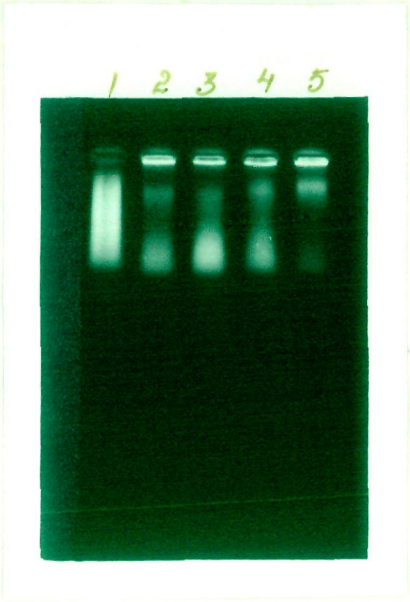


Fig.42. Binding of DNA-8MOP photoadduct by Z22 IgG as analyzed by gel retardation assay. DNA-8MOP photoadduct (0.5 ug) was incubated with buffer (lane 1) and increasing concentrations of anti-ZDNA (Z22) IgG (4 ug-lane 2; 8 ug-lane 3; 16 ug-lane 4 and 28 ug-lane 5) for 2 hr at 37°C and overnight at 4°C. Electrophoresis was carried out on 1% agarose gel for 2 hr at 40 volts.

shown to present Z- or Z-like features, the modified polymers were tested for their binding to human anti-DNA antibodies. Binding of three SLE autoantibodies (at 1:100 dilution of serum) with native DNA, DNA-8MOP and poly(dA-dT)-8MOP photoadducts has been presented in Fig. 43. The result of direct binding ELISA of these SLE autoantibodies showed significantly higher binding to modified polymers (DNA-8MOP and poly(dA-dT)-8MOP) as compared to native DNA.

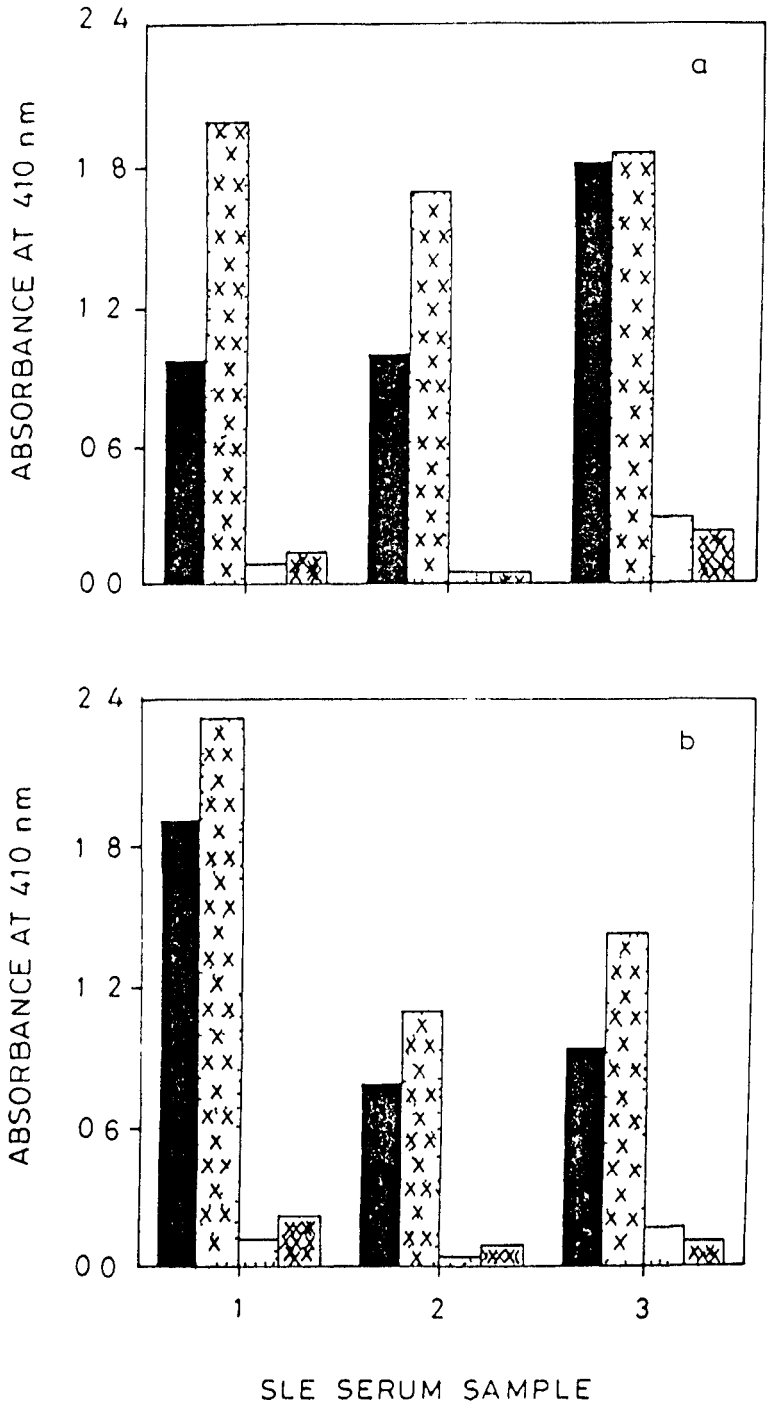


Fig.43(a). Direct binding ELISA of SLE sera with native DNA (■) and poly(dA-dT)-8MOP photoadduct (▨). Autoantibody negative control binding to native DNA (▩) and poly(dA-dT)-8MOP photoadduct (▨).

(b). Binding profile of SLE antibodies with native DNA (■) and DNA-8MOP photoadduct (▨). The small bars represent autoantibody negative control binding to native DNA (▩) and DNA-8MOP Photoadduct (▨).

IV DISCUSSION

Experimental records on immunogenicity of DNA in typical "B-conformation" have been puzzling and conventional wisdom implies that animals were tolerant to B-DNA which is most prevalent conformation under physiological conditions (Madaio et al., 1984; Pisetsky et al., 1990). By contrast, poly(dG).poly(dC), poly(dT-dC).poly(dG-dA), poly(dA).poly(dT), poly(dT).poly(dC), poly(A), poly(I), poly(G), double stranded RNA, triple helical DNA, DNA-RNA hybrids and cruciform junctions in RNA induce immunogen specific antibodies with little cross reactivity (Stollar, 1973, 1975, 1986, 1989; Anderson et al., 1988; Edgington and Stollar, 1992).

Under constraint conditions DNA can assume left handed conformation in which the Watson-Crick base pairing is preserved. The ribose-phosphate backbone in this double helix pursues a zig-zag course leading to the name Z-DNA for this left handed molecule. Covalent or noncovalent modification of B-DNA appears to be a prerequisite for antibody response (Braun and Lee, 1988). The left handed DNA (Z-DNA) is highly immunogenic (Lafer et al., 1981) and studies have pointed out its involvement in the regulation of transcription (Nordheim and Rich, 1983; Rich et al., 1984) and recombination events of the cell (Haniford and Pulleyblank, 1983; Kmiec et al., 1985). Moreover, these structures are interesting from physico-chemical point of view (Jovin and Soumpasis, 1987; Soumpasis, 1988; Doktycz et al., 1990; Suh et al., 1991; Lu et al., 1991). The B-form of poly(dG-dC).poly(dG-dC) is stabilized into Z-form by bromination under high salt (4 M NaCl) which alters its immunogenicity by several order of magnitude (Malfoy and Leng, 1981; Lafer et al., 1983; Thomae et al., 1983;

Zarling et al., 1984). Antibodies to Z-DNA have been used to search for base pair sequences capable of undergoing Z-transition (Nordheim et al., 1981, 1982; Lang et al., 1982; Lipps et al., 1983; Nordheim and Rich, 1983; Jovin et al., 1983). They have also been used to identify and isolate Z-DNA from E. coli (Thomae et al., 1983; Hoheisel and Pohl, 1987) and have served to titrate B- to Z-transition of polymer as a function of superhelical density (Stollar, 1989) and counterion or polyamine concentration (Thomas et al., 1988).

The genotoxic effects of DNA damage in living organisms include mutation, cancer, and lethality (Hanawalt and Sarasin, 1986). Most physical and chemical carcinogens interact primarily with DNA (Ames, 1979). The modifying effect of carcinogens on DNA could be covalent or non covalent. The best known covalent modifiers are antitumor drugs, alkylating agents and photoactivated species of furocoumarins (Waring, 1981). The covalent binding of furocoumarins to DNA is preceded by a complex intercalative step (Song and Tapley, 1979; Malagocka et al., 1990). The intercalated linear furocoumarins are capable of forming both mono- and diadducts upon UV-A (320-400 nm) irradiation (Dall'Acqua et al., 1979 b; Ortel et al., 1991). The exact effect of mono- or diadduct on the local structure of DNA is not known, but the crosslink substantially perturbs DNA base stacking, producing at least local changes in DNA conformation (Sherman et al., 1985). On the basis of psoralen-thymine monoadduct a model of crosslinked DNA (thymine-psoralen-thymine) has been proposed (Pearlman et al., 1985). It has been debated whether or not crosslink produces significant bends in DNA helix axis (Peckler et al., 1982; Sinden and Hagerman, 1984; Tomic et al., 1987; Zhen et al., 1988; Shi et al., 1988 b; Haran and Crothers, 1988).

The photosensitizing and photochemotherapeutic properties of furocoumarins are well documented (Parrish et al., 1974; Fitzpatrick et al., 1982). It has been reported that the formation of molecular complex is of preliminary importance in the formation of photoadduct and in the photobiological activity of various psoralens.

The present work involved the addition of 8-methoxypsoralen to calf thymus DNA (ctDNA) and poly(dA-dT) under UV-A light. As compared to native polymers, the ultraviolet spectra of DNA-8MOP and poly(dA-dT)-8MOP photoadducts demonstrated the photoaddition of 8-MOP resulting in the formation of photomodified conformers. The shifting of fluorescence emission maxima from 507 nm (for free 8-MOP) to 415 nm (DNA-8MOP and poly(dA-dT)-8MOP photoadducts) and a dramatic decline in fluorescence intensity of 8-MOP on photoconjugation with nucleic acid polymers is consistent with earlier work. The shift of maxima together with profound decline in intensity has been attributed to the conversion of fluorescent monoadducts to crosslinks.

The mono- or diadduct nature of photomodified conformers was analyzed by temperature induced melting of modified duplexes. The Tm values of photomodified polymers were found to increase to the extent of 29.5^{o}C and 5^{o}C as compared to unmodified conformers. The increased Tm values of modified duplexes indicate that diadduct (crosslink) has been formed as a result of photoreaction. Earlier, it has been shown that formation of covalent interstrand crosslinks progressively increases the denaturation temperature of photomodified nucleic acids and total percent increase in optical density (at 95^{o}C) becomes progressively smaller compared to unmodified conformer (Hurt et al., 1987). In confirmity with the above findings it could be inferred that after irradiation in presence of furocoumarins, a

fraction of DNA cannot be denatured. Furthermore, increased Tm values suggest that photoadducts are thermodynamically more stable. The results of thermal denaturation studies are in agreement with the work of Shi and Hearst (1986) who suggested that crosslinks are capable of stabilizing the helix from thermal denaturation.

The unwinding of the helix as a consequence of interstrand crosslink formation (Shi et al., 1988 b) was also confirmed by agarose gel electrophoresis of native and denatured photoadducts incubated with nuclease S1. Both native and denatured photoadducts revealed equal level of digestion, indicating that the denaturation of photoadducts did not cause further unwinding of the modified duplex. If formation of monoadducts would have occurred, enhanced digestion of denatured photoadducts with nuclease S1 would have been observed.

Hydroxyapatite column chromatography was used to detect interstrand crosslinks in DNA-8MOP and poly(dA-dT)-8MOP photoadducts by assessing the fraction of renaturable DNA after heat denaturation. The elution of denatured photoadducts as a major peak at the position of double stranded DNA indicates the formation of interstrand crosslinked (diadduct) species. Comparison of our data with those published earlier shows that the amount of crosslinks induced by 8-MOP lies in the same range. Hasan et al. (1991) reported that treatment of ctDNA with psoralen under UV-A light resulted in 74% crosslinks as analysed by hydroxyapatite column chromatography. Moreover, the work of Potter et al. (1980) have shown that mitochondrial DNA in presence of 4, 5', 8-trimethylpsoralen and UV light have produced crosslinks to such an extent that it appears completely double stranded under denaturing conditions in electron microscope.

Enzyme Bal 31 possess specific exonuclease activity which apparently identifies only crosslinks (Zhen et al., 1986). DNA exposed to Bal 31 showed complete digestion as revealed in polyacrylamide gel. Exposure of diadducts to Bal 31 for varying time periods had negligible effect on their digestion reiterating the interstrand crosslink formation in DNA exposed to 8-MOP and UV-A light. The analysis is based on the observation that the activity of some exonucleases is drastically reduced or blocked at sites of psoralen crosslinks in duplex DNA (Sage and Moustacchi 1987; Widmer et al., 1988).

In addition, DEAE Sephadex A 50 column chromatography of hydrolyzed photoadduct indicated the modification of only one pyrimidine base. Modification of thymine to the extent of 69 percent suggests that it is the most preferred substrate for photoreaction with 8-methoxypsoralen.

The covalent interstrand crosslinks isolated through hydroxyapatite column, after thorough chracterization were injected in rabbit and goat. The induced antibodies against DNA-8MOP an poly(dA-dT)-8MOP adducts were found to be precipitating in nature as revealed by immunodiffusion and quantitative precipitin assay. The results indicate that the crosslinks are also capable of inducing high antibody response like many conformationally altered immunogenic DNA molecules (Hasan and Ali, 1990). The high binding of induced antibodies towards respective immunogens point out the native epitope modification of ctDNA and poly(dA-dT) on photoaddition of 8-methoxypsoralen which has rendered the molecule highly immunogenic. Of both the immunogens, the poly(dA-dT)-8MOP crosslinks were comparatively more immunogenic than DNA-8MOP crosslinks as revealed by high titer (>1:51200 vs >1:6400) antibodies. This could be due

to sequence specificity exhibited by psoralens in their photobinding to nucleic acids. At the dinucleotide level, (TpA) base pair steps are the most reactive (Kanne et al., 1982 b). The context of (TpA) base pair in nucleic acids strongly influences their photoreactivity to psoralens since isolated (TpA) steps embedded in GC-rich domains are much less reactive than (TpA) steps in AT-rich domains of poly(dA-dT). In calf thymus DNA only 6.1% of the base pair steps are (TpA) steps (Fasman, 1976).

The IgG isolated from immune sera was characterized by absorption ratio (278/251) measurements and fractions having ratio of less than 2.5 were further purified through Sephadex G 200 column to remove contaminating proteins. The homogeneity of isolated IgG was also confirmed by a single band movement in polyacrylamide gel electrophoresis.

The G 200 purified immune IgG from anti-DNA-8MOP and anti-poly(dA-dT)-8MOP sera retained their strong binding with respective immunogens. The high binding of anti-poly(dA-dT)-8MOP IgG further substantiates that poly(dA-dT)-8MOP photoadduct was a better immunogen as compared to ctDNA modified under identical conditions, as each base pair in poly(dA-dT).poly(dA-dT) possesses the potential of crosslink formation (Gasparro and Santella, 1988).

The specificity of anti-photoadduct antibodies was probed by competitive binding assay. Binding data revealed that both anti-DNA-8MOP and anti-poly(dA-dT)-8MOP antibodies were highly specific towards typical epitope of immunogen. The affinity of anti-poly(dA-dT)-8MOP antibodies was comparatively higher as evident from low competitor requirement to eliminate 50% of antibody binding. Direct binding and competition data indicate the

specific binding characteristics of induced antibodies. These results support the postulate that psoralen modification alters the antigenic determinant of DNA, producing antibodies specific for psoralen modified DNA (Santella et al., 1985; Sundquist et al., 1987). Presumably, any unmodified portions of duplex are rapidly degraded during antigen processing so that the immune response is directed to DNA-drug adducts.

The specific binding of anti-poly(dA-dT)-8MOP IgG was also analyzed by band shift assay in agarose gel. The formation of antibody-immunogen complex as indicated by retarded mobility conclusively demonstrates the specific nature of induced antibodies.

The immunogen specific antibodies from total IgG molecules purified from anti-DNA-8MOP immune sera were passed through immunoaffinity column of DNA-8MOP linked to polylysyl-Sepharose 4B. The specificity of immunoaffinity purified antibodies was significantly increased when used in competition ELISA. The non-reactivity of immunoaffinity purified antibodies incubated with ctDNA, ssDNA, and RNA reflects that the induced antibodies are conformation specific, recognizing specifically the unique conformation at the site of crosslink.

Calf thymus DNA photomodified with psoralen, besides 8-MOP, was found to possess epitopes typical of poly(dA-dT)-8MOP. The conclusion is based on the inhibition of anti-poly(dA-dT)-8MOP IgG - immunogen interaction by DNA-8MOP and DNA-psoralen to the level of nearly 55 and 49 percent respectively. The epitope sharing of DNA-8MOP and DNA-psoralen photoadduct with poly(dA-dT)-8MOP was confirmed by gel retardation assay. However the same immune IgG incubated with native DNA and poly(rG).poly(dC) did not result in immune complex

formation and rules out the possible presence of B- / A- or A- like conformation on photocross-linking site.

The data of quantitative precipitin assay was analyzed by scatchard and Langmuir isotherm plots and antibody affinity was computed to be 1.13×10^{-9} M and 6.80×10^{-10} M for anti-DNA-8MOP and anti-poly(dA-dT)-8MOP IgG respectively. The affinity constants clearly indicate that the photoadducts were highly immunogenic inducing immunogen specific antibodies.

The conformational perturbations in DNA molecule caused by chemical modification can be precisely determined by both highly sensitive physico-chemical techniques and monoclonal antibodies. Furthermore, monoclonal antibodies are non destructive models of protein-nucleic acid interactions (Anderson et al., 1988). The monoclonal anti-ZDNA antibody (Z22) is an immunochemical marker for Z-conformation, such immunochemical probes can detect these structures in either oligonucleotides or in larger mammalian genome (Sanford et al., 1988). The high specificity of Z22 IgG for Z-DNA is evident from inhibition of Z22 binding by low concentration of prototype Z-DNA (i.e. high salt brominated poly(dG-dC)). Taking advantage of specific Z-epitope recognition, the Z22 was utilized to detect its binding with poly(dA-dT)-8MOP and DNA-8MOP photocrosslinks. The gel retardation and competitive binding assay of Z22 antibody with poly(dA-dT)-8MOP and DNA-8MOP photoadducts demonstrated high specific binding of anti-ZDNA IgG with these antigens. Binding of anti-poly(dA-dT)-8MOP IgG with DNA brominated under high salt as well reflects that the photoadducts represent epitopes which were typical of Z- or Z- like conformation. The modified polymer in the present study induced high titer antibodies which were specifically recognizing the Z- or Z- like epitopes on brominated DNA. Furthermore, it could

be inferred that Z- epitopes on poly(dA-dT)-8MOP crosslink rendered the molecule highly immunogenic. Native DNA brominated under high salt has been shown to present features of Z- or Z- like conformation inducing high titer and highly specific antibodies (Hasan and Ali, 1990). These studies confirmed the presence of Z- or Z-like epitopes on crosslinks as Z22 antibody does not recognize other nucleic acid conformers.

Based on the above studies the following conclusions can be drawn:

1. Ultraviolet, fluorescence and nuclease sensitivity assay demonstrate the formation of crosslinks in DNA and poly(dA-dT) as a result of 8-MOP photoaddition.
2. Both the photomodified conformers are thermodynamically more stable as compared to their native forms.
3. Thymine acts as a major substrate for photoactivated 8-MOP binding.
4. That the modified DNA polymers are conformationally distinct from B-DNA as proved by its potentiality to generate high titer immunogen specific antibodies.
5. The binding of Z22 antibody to poly(dA-dT)-8MOP and DNA-8MOP crosslinks (diadducts) is an indicative of Z- or Z- like structure at the site of crosslink.

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